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SOME OBSERVATIONS ON THE LETHAL EFFECTS OF VARIOUS CHEMICALS AGAINST THE FREE-LIVING STAGES OF SCLEROSTOMES (NEMATODA)*

BY

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After the discovery of the life history of the hookworms of man, control measures included the treatment of night-soil with various readily available chemicals, such as sodium chloride, ammonium sulphate, sodium nitrate, other fertilizers, and with urine to kill the eggs and free-feeding larvae.

In 1933 work was begun at the Institute of Parasitology of McGill University† with a view to finding chemicals which could be used to kill the free-living stages of Sclerostomes in manure heaps. It was soon found that this only dealt with one aspect of the problem, and left the control of larvae in stables and sheds and on pastures unsolved; just as treating night-soil leaves the problem of larvae on the wood and metal of latrines, and faeces in the fields as sources of hookworm infection. For these reasons a much wider range of chemicals was tested. It was also suggested that information on the types of chemicals which are lethal to the free-living stages of bursate nematodes might suggest types of chemicals which would be lethal to some of the nematodes which are parasitic on plants.

In 1946 the work was continued at the Department of Zoology of Edinburgh University.‡ The range of chemicals tested was again considerably widened, and included many which were of only theoretical interest, in order to test some theories which had been formulated on the chemical constitution of substances which are lethal. For this purpose appropriate chemicals were made at the Heriot-Watt College.‡ Furthermore it was hoped that indications might be obtained on the types of chemical which could be used as anthelmintics; especially as anthelmintics against the immature forms of bursate nematodes in the abomasum and small intestine. At present there is no satisfactory anthelmintic against many immature nematodes, a want which constitutes a very serious deficiency in prophylactic dosing of farm animals.

Wright and Schaffer (1932) tested a series of chlorinated aliphatic hydrocarbons against hookworms in dogs and found that anthelmintic effect depended upon solubility in water, length of carbon chain, and the position of the halogen in the molecule. During recent years many chemicals have been tested as anthelmintics in laboratory animals and against plant nematodes. In addition, numerous chemicals

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‡ Financed by the Agricultural Research Council of Great Britain.

have been tested *in vitro* by Lamson and Brown (1936) and Baldwin (1943, 1948) with *Ascaris lumbricoides*, by Chance and Mansour (1949) with *Fasciola hepatica*, and by Duguid and Heathcote (1950) with *Moniezia expansa*. These screening tests have given many indications of the types of chemicals which are lethal to various adult helminths. Levine (1949, 1951a and b) screened numerous chemicals against Sclerostome larvae in faeces, from which he was able to draw some conclusions on the types of chemicals which are lethal to the free-living stages of bursate nematodes.

However, at present there is no thoroughly satisfactory anthelmintic for some of the common bursate nematodes of domestic animals; there is no chemical which could be recommended to kill bursate larvae in latrines, stables, sheds, or pens, and there is no practical method of killing larvae on pastures.

The results given in this paper can almost be considered as *in vivo* tests against the free-living stages of bursate nematodes, and, in some respects, as *in vitro* anthelmintic tests against the immature parasitic forms of some genera. The fact that the tests of the chemicals as ovicides and larvicides were in small cultures in a confined space at a comparatively high temperature (25° C.) gave the chemicals greater opportunities of being effective than would occur in practice.

Method

Chemicals have been tested by adding them to 40 g. of horse faeces within five hours of the faeces being passed. When the chemicals were tested in the dry state, they and the faeces were mixed and tied up in butter muslin or cheese cloth, and put into glass preserving jars of about 550 c.c. capacity, fitted with lids without rubber rings. Chemicals in solution or suspension were poured slowly over the faeces which had previously been tied up in butter muslin and placed in the jars. A few chemicals were tested in 80, 16, and 10 g. cultures, contained in proportionately sized jars; the smaller jars were fitted with shives with a small hole bored in them. The cultures were kept for a minimum of ten days, but usually longer, in a constant temperature room, at $25 \pm 2°$ C., which gave the free-living stages more than sufficient time to reach the infective stage. Untreated control cultures were always made. The larvae, if any, were extracted by soaking the cultures in warm water in 8-in. glass funnels. To collect any larvae on the walls of the jars, they were filled with water, which was also poured into the funnels. Two or three days later more warm water was added to the funnels. Six to ten days later the bottom 50 c.c. of each funnel was drawn off into an Erlenmeyer flask. The larvae in this fluid were then counted on a squared glass dish with the help of a dissecting microscope; if the larvae were numerous a dilution technique was used before counting—control cultures usually contained several thousand larvae. This technique has already been described in greater detail by Parnell (1936, 1938).

When a chemical was first tested cultures were made in a wide range of concentrations. After these preliminary cultures had shown the probable quantity of chemical required to kill the free-living stages, cultures were made in duplicate above, at, and below the indicated lethal quantities; and single cultures were made with still greater and lesser amounts of chemical. The final results, therefore, were usually based on three cultures for the quantities of chemical approximating to the lethal percentage, and in addition there were series of one or two cultures with considerably more and considerably less chemical.

The majority of the chemicals were tested by being mixed dry or undiluted in the faeces, and, if soluble in water, were also tested as aqueous solutions; a few chemicals were tested as aqueous suspensions. Amounts up to 20 g. of solid and of 25 c.c. of liquid were

added to 40 g. of faeces; 25 c.c. of fluid is about the maximum quantity that 40 g. of fresh horse faeces will absorb. The more effective the chemicals were the more accurately were their values assessed. When very small amounts of chemical were applied the difference between quantities was only 0.001 g. or 0.001 c.c., but with larger quantities the difference was as much as 4 g. or 5 c.c. Therefore, it follows that with the less lethal chemicals smaller percentages might have been effective. Similarly, if more than three cultures had been made, sometimes the arbitrary 90 and 99.9 reduction percentages might have been slightly different; this is especially true where some larvae avoided the action of the chemical in one culture.

In the following Tables, " very concentrated solutions " implies 1 g. of chemical to 2 or 4 c.c. of water, " concentrated solutions " were 1 g. of chemical to 8 or 20 c.c. of water, " moderately concentrated solutions " were 1 g. of chemical to 50 or 100 c.c. of water, " moderately dilute solutions " were 1 g. of chemical to 200, 300, 400, or 500 c.c. of water, " dilute solutions " were 1 g. of chemical to 600, 800, 1,000, 1,250, and 1,500 c.c. of water, " very dilute solutions " were 1 g. of chemical to 2,000, 2,500, 3,000, 4,000, 5,000, 6,000, and 8,000 c.c. of water, and " extremely dilute solutions " were 1 g. of chemical to 10,000, or more, c.c. of water. The most effective results are quoted for each group of solutions. A few chemicals were also measured as gases.

The description " third stage larvae " has been used in most places in this paper in preference to infective larvae, because it is probable that some of the larvae which reached the third stage in treated cultures, although alive and often active, had been damaged sufficiently by the chemical to reduce considerably their ability to infest a host.

Results

Table I gives the percentages by weight of eighty inorganic chemicals and Table II gives similar figures for nearly two hundred organic chemicals and other substances which gave a 90 and a 99.9 per cent reduction in the number of third stage larvae found in the cultures. Forty-five chemicals and other materials are listed which have no lethal value or such a low value that it was not determined. Reductions in the percentage of larvae recovered caused by alteration in the consistency of the cultures have been ignored, as far as possible. Sometimes insufficient chemical was added to the cultures to enable the lethal value to be determined; this is indicated in Tables I, II, and III by an asterisk (*); where the quantity of solid or undiluted chemical was more or less than 50 per cent it is shown by including the percentage in brackets below the asterisk. When the chemical was in solution the maximum amount applied has been the percentage in 25 c.c. added to 40 g. faeces.

Only the most lethal chemicals were tested as very dilute or extremely dilute solutions; that is, in solutions containing only one gramme of chemical in 2,000 c.c., or more, of water. Table III lists those chemicals which were effective in " very and extremely dilute solutions," and shows the percentages of chemical to faeces required for 90 and 99.9 per cent reductions in the average numbers of third stage larvae recovered.

Although free-feeding larvae are usually more easily killed by unfavourable conditions than are infective larvae, some of the larvae have been able to develop to the third stage before being killed in cultures treated with some chemicals. A few of these chemicals have killed all these larvae, others have only killed some. Chemicals which fairly frequently showed this delayed killing action are listed in Table IV. Often the percentage of chemical necessary to produce this phenomenon varied considerably, depending on whether the chemical was added as a solid or

TABLE I

INORGANIC CHEMICALS

Chemical	Percentage of chemical to fresh faeces required to reduce the number of larvae by											
	90% when applied						99.9% when applied					
	Dry or undiluted	In very concentrated solutions	In concentrated solutions	In moderately concentrated solutions	In moderately dilute solutions	In dilute solutions	Dry or undiluted	In very concentrated solutions	In concentrated solutions	In moderately concentrated solutions	In moderately dilute solutions	In dilute solutions
Sodium compounds:												
Sodium hydroxide	1.9	3.0	1.9	1.25	—	—	2.5	4.0	2.5	—	—	—
Sodium bicarbonate	7.5	—	6.0	—	—	—	20.0	—	7.5	—	—	—
Sodium fluoride	0.5	—	0.9	0.2	0.08	0.06	0.8	—	0.9	0.4	0.25	*
Sodium chloride	2.5	4.1	1.9	*	—	—	5.0	4.1	2.5	*	—	—
Sodium iodide	0.02	0.2	0.12	0.1	0.025	0.02	0.19	0.9	0.37	0.13	0.05	0.04
An aqueous solution of sodium hypochlorite with 1.2% available chlorine	19.0	*	*	—	—	—	25.0	*	*	—	—	—
Sodium chlorate	3.75	4.0	2.5	—	—	—	6.25	5.75	6.0	—	—	—
Sodium sulphate	12.5	14.2	*	—	—	—	20.0	19.0	*	—	—	—
Sodium sulphite	10.0	8.6	*	—	—	—	15.0	25.0	*	—	—	—
Sodium sulphide	10.0	7.2	7.4	—	—	—	12.5	14.4	*	—	—	—
Sodium nitrate	3.7	2.9	2.3	—	—	—	6.2	5.8	3.1	—	—	—
Trisodium phosphate	6.2	7.2	6.0	—	—	—	12.25	12.5	*	—	—	—
Sodium tetraborate	0.63	—	0.9	0.4	—	—	3.75	—	1.8	1.0	—	—
Sodium silicofluoride	1.25	—	—	0.3	0.06	0.06	3.7	—	—	0.5	0.2	*
Potassium compounds:												
Potassium hydroxide	2.5	3.0	2.5	*	—	—	3.75	4.6	4.5	*	—	—
Potassium carbonate	7.5	5.0	4.5	—	—	—	7.5	7.5	7.5	—	—	—
"Muriate of potash," fertilizer	3.7	4.3	3.7	—	—	—	7.5	5.7	6.0	—	—	—
Potassium iodide	0.025	0.6	0.3	0.13	0.04	0.015	0.075	1.2	0.5	0.25	0.06	0.05
Potassium chlorate	15.0	6.2	3.1	*	—	—	35.0	15.5	6.0	*	—	—
Potassium iodate	0.05	—	0.25	0.1	0.05	0.025	0.62	—	0.5	0.19	0.08	0.08
Potassium sulphate	10.0	11.5	7.5	—	—	—	30.0	20.0	*	—	—	—
Potassium nitrate	3.7	4.3	3.0	—	—	—	12.5	4.3	4.5	—	—	—
Potassium cyanate	1.25	1.15	0.9	0.5	*	—	1.9	1.5	1.8	0.75	*	—
162 parts potassium cyanate + 96 parts ammonium carbonate	1.25	1.5	1.25	0.5	—	—	1.9	1.5	1.6	*	—	—
Potassium permanganate	7.5	—	*	—	—	—	25.0	—	*	—	—	—
Ammonia and ammonium compounds:												
Aqueous ammonia (containing 27% NH_3)	1.3	1.5	2.1	*	—	—	1.3	2.1	2.1	*	—	—
Ammonium carbonate	1.9	1.6	1.4	1.0	—	—	2.5	1.9	1.8	*	—	—
Ammonium chloride	1.9	2.1	1.1	1.2	—	—	3.7	4.0	1.1	*	—	—
Ammonium iodide	0.08	0.44	0.19	0.1	0.033	0.024	0.5	1.8	0.5	0.19	0.08	0.09
Ammonium sulphate	5.0	4.3	3.0	—	—	—	10.0	7.2	6.0	—	—	—
Ammonium sulphide (15% aqueous solution)	6.2	4.2	5.5	—	—	—	10.0	6.2	7.0	—	—	—
Ammonium sulphamate	0.82	1.5	0.6	0.5	0.2	—	2.5	2.4	1.85	0.62	*	—
Ammonium nitrate	1.9	3.9	1.9	—	—	—	3.7	4.0	3.0	—	—	—
"Nitro chalk"	6.2	5.0	3.1	—	—	—	7.5	7.2	7.5	—	—	—
"Calnitro"	2.5	4.3	2.5	—	—	—	6.2	7.2	6.0	—	—	—
Diammonium phosphate	3.7	4.3	2.5	—	—	—	6.2	7.2	6.0	—	—	—
Ammonium thiocyanate	0.5	0.8	0.6	0.2	0.13	—	1.9	2.1	1.5	0.5	*	—
Copper compounds:												
Cuprous chloride	3.75	—	—	—	—	—	6.2	—	—	—	—	—
Cupric chloride	1.9	2.5	1.6	0.6	—	—	3.75	3.1	3.1	*	—	—
Cupric sulphate	6.2	4.3	4.5	*	—	—	15.0	8.6	*	*	—	—
Cupric nitrate	3.7	2.5	1.6	1.2	—	—	6.2	4.2	3.1	*	—	—
Magnesium compounds:												
Magnesium chloride	6.2	7.2	6.0	—	—	—	12.5	8.6	7.5	—	—	—
Magnesium sulphate	17.5	15.5	*	—	—	—	35.0	*	*	—	—	—
Kainite (high grade)	3.7	2.9	2.2	—	—	—	5.0	4.0	7.5	—	—	—
Magnesium borate	0.5	—	—	—	—	—	1.9	—	—	—	—	—

TABLE I—*continued*

Chemical	Percentage of chemical to fresh faeces required to reduce the number of larvae by											
	90% when applied						99·9% when applied					
	Dry or undiluted	In very concentrated solutions	In concentrated solutions	In moderately concentrated solutions	In moderately dilute solutions	In dilute solutions	Dry or undiluted	In very concentrated solutions	In concentrated solutions	In moderately concentrated solutions	In moderately dilute solutions	In dilute solutions
Calcium compounds:												
Bleaching powder	5.0	4.3	3.5	—	—	—	7.5	7.5	5.5	—	—	—
Calcium nitrate	3.7	4.3	3.0	—	—	—	10.0	5.7	7.5	—	—	—
"Calurea"	1.2	0.9	1.2	1.2	—	—	3.7	1.4	1.2	1.2	—	—
16% superphosphate	35.0	—	—	—	—	—	50.0	—	—	—	—	—
20% superphosphate	15.0	—	—	—	—	—	20.0	—	—	—	—	—
Calcium arsenate	1.0	—	—	—	—	—	20.0	—	—	—	—	—
Calcium cyanide	0.05	—	—	—	—	—	0.19	—	—	—	—	—
Calcium cyanamide (powdered)	1.9	1.8	0.6	0.8	—	—	1.9	4.8	1.8	*	—	—
Calcium cyanamide (granular)	1.2	—	—	—	—	—	2.5	—	—	—	—	—
Calcium chloroacetate	1.25	1.15	0.9	0.25	0.25	—	3.75	5.75	1.85	1.0	*	—
Zinc compounds:												
Zinc chloride	1.9	1.6	1.25	0.5	—	—	3.75	4.2	1.9	1.0	—	—
Zinc iodide	0.13	0.35	0.19	0.06	0.02	0.02	0.63	1.0	0.3	0.13	0.05	0.05
Zinc sulphate	1.3	2.5	1.25	0.5	—	—	15.0	5.8	3.1	*	—	—
Zinc sulphide	50.0	—	—	—	—	—	*	—	—	—	—	—
Mercury compounds:												
Mercurous chloride	0.037	—	—	—	—	—	0.1	—	—	—	—	—
Mercuric chloride	0.025	—	0.025	0.008	0.008	0.009	0.062	—	0.1	0.025	0.019	0.017
Ethyl-mercuric chloride	0.004	—	—	—	—	—	0.025	—	—	—	—	—
Ethoxy-ethyl-mercuric chloride	0.025	—	—	—	0.006	0.008	0.37	—	—	—	0.04	0.03
Mercuric iodide	0.12	—	—	—	—	—	3.7	—	—	—	—	—
A seed dressing containing mercury alkoxy-ethyl	1.25	—	—	—	—	—	2.5	—	—	—	—	—
Boron compound:												
Ortho-boric acid	0.63	—	—	—	—	—	1.9	—	—	—	—	—
Carbon compound:												
Carbon disulphide	0.32	—	—	—	—	—	1.6	—	—	—	—	—
Iodine:												
Iodine	0.08	—	—	—	—	—	0.38	—	—	—	—	—
10% colloidal iodine in vegetable oil	0.8	—	—	—	—	—	7.5	—	—	—	—	—
0.12% iodine in oily medium	32.0	—	—	*	—	—	62.0	—	—	*	—	—
"Iodine vermicide"—containing 16% iodine	1.9	—	0.3	0.4	0.08	*	5.0	—	0.08	0.8	0.3	*
Manganese compounds:												
Manganous chloride	2.5	3.1	2.25	1.25	—	—	6.2	6.2	3.0	*	—	—
Manganous sulphate	7.5	4.0	1.5	—	—	—	12.5	8.5	2.5	—	—	—
Iron compounds:												
Ferrous chloride	3.75	2.5	1.85	*	—	—	3.75	5.0	3.1	*	—	—
Ferrous iodide	0.08	—	—	0.12	0.06	0.05	0.8	—	—	0.38	0.1	*
Ferrous sulphate	3.75	4.0	2.5	—	—	—	10.0	7.0	6.0	—	—	—
Ferric chloride	3.75	2.3	2.1	*	—	—	3.75	2.9	3.1	*	—	—
Ferric sulphate	3.7	—	—	—	—	—	5.0	—	—	—	—	—
Cobalt compound:												
Cobalt chloride	3.75	2.9	1.85	0.5	—	—	6.25	5.7	2.45	*	—	—
Nickel compound:												
Nickel chloride	3.75	2.8	1.25	0.5	0.17	—	10.0	5.8	2.5	1.25	0.3	—

TABLE II

ORGANIC CHEMICALS

Chemical	Percentage of chemical to fresh faeces required to reduce the number of larvae by 90% when applied: Dry or undiluted	In very concentrated solutions	In concentrated solutions	In moderately concentrated solutions	In moderately dilute solutions	In dilute solutions	99.9% when applied: Dry or undiluted	In very concentrated solutions	In concentrated solutions	In moderately concentrated solutions	In moderately dilute solutions	In dilute solutions
ALIPHATIC COMPOUNDS												
Paraffins:												
44.3% Naphtha + 5.3% oleic acid + 1.5% Teepol X + 0.6% NaOH and water ..	0.23	—	0.2	0.23	0.23	—	0.35	—	0.44	0.34	0.29	—
Halogen derivatives of paraffins:												
Methyl bromide	0.009	—	—	—	—	—	0.02	—	—	—	—	—
Methyl iodide	0.006	—	—	0.006	0.004	0.003	0.012	—	—	0.012	0.007	0.007
Ethyl chloride	4.5	—	—	—	*	—	*(57.5)	—	—	—	—	*
Ethyl bromide	0.15	—	—	—	—	—	0.4	—	—	—	—	—
Ethyl iodide	0.02	—	—	—	0.024	0.015	0.07	—	—	—	0.035	0.04
n-Propyl bromide	0.11	—	—	—	*	—	0.25	—	—	—	*	—
n-Propyl iodide	0.033	—	—	—	—	0.03	0.066	—	—	—	—	0.09
n-Butyl chloride	0.74	—	—	—	—	—	1.12	—	—	—	—	—
n-Butyl bromide	0.1	—	—	—	—	—	0.32	—	—	—	—	—
tert.-Butyl bromide	1.2	—	—	—	—	—	1.5	—	—	—	—	—
n-Amyl bromide	0.3	—	—	—	—	—	0.6	—	—	—	—	—
n-Hexyl bromide	0.072	—	—	—	—	—	0.16	—	—	—	—	—
n-Heptyl bromide	0.043	—	—	—	—	—	0.14	—	—	—	—	—
n-Octyl bromide	0.055	—	—	—	—	—	0.14	—	—	—	—	—
Methylene dichloride	0.67	—	—	—	—	—	2.5	—	—	*	—	—
Methylene dibromide	0.47	—	—	0.77	0.5	—	0.63	—	—	1.2	0.8	—
Methylene diiodide	0.008	—	—	0.04	0.012	0.004	0.02	—	—	0.23	0.07	0.03
Ethylene dichloride	0.5	—	—	*	*	—	1.0	—	—	*	*	—
Ethylidene dichloride	1.17	—	—	—	*	—	2.2	—	—	—	*	—
10% methyl bromide + 67.5% ethylene dichloride, 22.5% carbon tetrachloride	0.47	—	—	—	—	—	0.78	—	—	—	—	—
Ethylene dibromide	0.08	—	—	—	—	—	0.08	—	—	—	—	—
1 : 3-Dichloropropylene + 1 : 2-Dichloropropane + higher chlorides (D.D. mixture) ..	0.01	—	—	—	—	—	0.019	—	—	—	—	—
Dichloropentanes	0.27	—	—	—	—	—	0.54	—	—	—	—	—
Chloroform	1.2	—	—	—	*	—	3.75	—	—	—	*	—
Bromoform	0.18	—	—	—	0.22	0.15	0.29	—	—	—	0.36	0.22
Iodoform	0.075	—	—	—	—	—	0.18	—	—	—	—	—
Chloropicrin	0.02	—	—	—	—	—	0.02	—	—	—	—	—
1 : 1 : 1-Trichloroethane	1.1	—	—	—	—	—	2.5	—	—	—	—	—
Carbon tetrachloride	1.3	—	—	—	—	—	5.9	—	—	—	—	—
Carbon tetrabromide	0.019	—	—	—	—	—	0.025	—	—	—	—	—
1 : 1 : 2 : 2-Tetrachloroethane ..	0.1	—	—	—	—	—	0.13	—	—	—	—	—
Pentachloroethane	0.21	—	—	—	—	—	0.42	—	—	—	—	—
Hexachloroethane	0.12	—	—	—	—	—	0.25	—	—	—	—	—
Halogen derivatives of ethylene:												
Dichlorethylene (*cis*)	0.65	—	—	—	—	—	1.06	—	—	—	—	—
Dichlorethylene (*trans*)	0.62	—	—	—	—	—	1.56	—	—	—	—	—
Trichlorethylene	0.36	—	—	—	—	*	0.72	—	—	—	—	*
Perchloroethylene	0.31	—	—	—	—	—	0.31	—	—	—	—	—
Alcohols:												
Methyl alcohol	4.0	2.65	3.4	—	—	—	5.0	6.0	*	—	—	—
Ethyl alcohol	3.0	2.0	2.8	—	—	—	5.0	3.3	4.4	—	—	—
n-Propyl alcohol	1.5	1.3	1.7	*	—	—	1.5	1.6	2.2	*	—	—
iso-Propyl alcohol	1.5	1.6	1.6	*	—	—	3.0	2.0	2.3	*	—	—
n-Butyl alcohol	0.66	—	0.95	0.8	—	—	1.5	—	1.7	1.0	—	—
iso-Butyl alcohol	0.8	—	0.9	0.97	—	—	3.0	—	1.1	*	—	—
tert.-Butyl alcohol	1.5	1.0	1.1	*	—	—	1.5	1.3	1.6	*	—	—
Ethers:												
Ether	1.8	—	1.3	—	—	—	3.6	—	*	—	—	—
Dichlorodiethyl ether	0.06	—	—	0.05	0.05	0.05	0.1	—	—	0.09	0.11	0.11
50% β-butoxy-β-thiocyanodiethyl ether + 50% petroleum distillate of the kerosene type	0.46	—	—	—	—	—	0.75	—	—	—	—	—

TABLE II—*continued*

Chemical	Percentage of chemical to fresh faeces required to reduce the number of larvae by											
	90% when applied						99.9% when applied					
	Dry or undiluted	In very concentrated solutions	In concentrated solutions	In moderately concentrated solutions	In moderately dilute solutions	In dilute solutions	Dry or undiluted	In very concentrated solutions	In concentrated solutions	In moderately concentrated solutions	In moderately dilute solutions	In dilute solutions
Sulphides:												
Tetramethyl thiuram monosulphide	1.9	—	0.9	0.25	0.19	—	40.0	—	1.9	1.25	*	—
46% tetramethyl thiuram disulphide	5.0	—	0.9	0.2	0.06	0.1	7.5	—	1.8	0.6	0.16	*
Formaldehyde and derivative:												
Formalin	2.0	0.7	0.5	0.27	0.2	—	2.0	1.4	1.2	0.55	0.35	—
Hexamethylene tetramine	7.5	4.5	*	—	—	—	12.5	18.0	*	—	—	—
Ketone:												
Acetone	3.6	3.3	4.4	—	—	—	8.0	6.6	5.5	—	—	—
Soaps:												
Hard soap (Castile)	—	11.0	—	—	—	—	—	*	—	—	—	—
Soft soap	—	*	—	—	—	—	—	*	—	—	—	—
Esters:												
Ethyl iodoacetate	0.06	—	—	—	—	—	0.19	—	—	—	—	—
Dodecyl thiocyanate	3.75	—	—	—	—	—	60.0	—	—	—	—	—
50% mixed thiocyanates + 50% petroleum distillate of the kerosene type	0.46	—	—	—	—	—	0.75	—	—	—	—	—
Hexaethyl tetraphosphate	3.8	—	—	*	—	—	6.2	—	—	*	—	—
Di-ethyl-*p*-nitrophenylthiophosphate	0.8	—	—	—	—	—	39.0	—	—	—	—	—
Sodium alkyl sulphates (Teepol X)	10.5	—	1.6	*	—	—	66.0	—	*	*	—	—
Amines:												
25% dimethylamine	3.5	1.9	2.7	*	—	—	3.5	3.1	5.2	*	—	—
Cetyltrimethylammonium bromide	3.75	—	1.8	0.75	—	—	30.0	—	*	*	—	—
20% solution of alkyl ammonium bromides	—	—	1.5	—	—	—	—	—	*	—	—	—
10% alkyl-dimethyl-benzyl ammonium chlorides	25.0	6.2	2.4	0.6	—	—	37.5	*	*	*	—	—
Allyl compounds:												
Allyl isothiocyanate	0.0015	—	—	—	0.002	0.002	0.0025	—	—	—	0.004	0.003
Allyl chloride	0.1	—	—	—	—	—	0.23	—	—	—	—	—
Allyl bromide	0.007	—	—	—	—	—	0.026	—	—	—	—	—
Allyl iodide	0.0012	—	—	—	—	—	0.003	—	—	—	—	—
Allyl alcohol	0.05	0.05	0.05	0.03	0.05	0.07	0.16	0.08	0.1	0.08	0.11	*
Dihydric alcohol and derivative:												
2-Ethyl-hexanediol-1:3	10.0	—	—	—	—	—	19.0	—	—	—	—	—
"Lubrol W."	—	—	1.0	1.0	—	—	—	—	*	*	—	—
Keto-acid:												
Pyruvic acid	6.3	3.1	1.9	1.55	—	—	15.7	5.3	3.0	*	—	—
Carbonic acid derivatives:												
80% zinc dimethyldithiocarbamate	*	—	1.5	0.4	0.16	—	*	—	3.1	0.6	*	—
80% ferric dimethyldithiocarbamate	1.25	—	0.9	0.4	0.2	—	*	—	2.4	0.6	*	—
Urea	0.8	0.7	0.6	0.5	—	—	0.8	0.7	0.9	0.6	—	—
Guanidine hydrochloride	—	—	0.9	0.3	—	—	—	—	1.5	0.6	—	—
Methylguanidine sulphate	—	—	0.9	0.5	—	—	—	—	1.8	1.2	—	—
as-Dimethylguanidine sulphate	—	—	0.9	0.5	—	—	—	—	1.5	1.0	—	—
Diphenylguanidine	—	—	2.5	0.6	—	—	—	—	*	*	—	—
Creatine	—	—	—	0.9	—	—	—	—	—	*	—	—
Creatinine	—	—	0.9	1.0	—	—	—	—	*	1.2	—	—
Thiourea	0.1	—	0.3	0.1	0.06	0.06	0.4	—	1.8	0.3	0.2	*
Potassium xanthogenate	0.5	0.7	0.9	0.2	0.16	—	1.9	2.4	1.2	0.6	*	—
Esters of dibasic acids:												
Butyl mesityl oxalic ester	10.4	—	—	—	—	—	13.0	—	—	—	—	—
Butyl mesityl oxide oxalate	19.0	—	—	—	—	—	38.0	—	—	—	—	—

TABLE II—*continued*

Chemical	Percentage of chemical to fresh faeces required to reduce the number of larvae by											
	90% when applied						99.9% when applied					
	Dry or undiluted	In very concentrated solutions	In concentrated solutions	In moderately concentrated solutions	In moderately dilute solutions	In dilute solutions	Dry or undiluted	In very concentrated solutions	In concentrated solutions	In moderately concentrated solutions	In moderately dilute solutions	In dilute solutions
Ureides and purine derivatives:												
Barbituric acid	3.75	—	—	1.25	*	—	17.5	—	—	*	*	—
Allantoin	1.25	—	—	—	—	—	*	—	—	—	—	—
Xanthine	7.5	—	—	—	*	—	*	—	—	—	*	—
Guanine	10.0	—	—	—	—	—	(10.0) * (15.0)	—	—	—	—	—
Carbocyclic Compounds												
Hydrocarbons:												
Benzene	0.56	—	—	—	—	—	0.72	—	—	—	—	—
Toluene	0.16	—	—	—	—	—	0.86	—	—	—	—	—
Xylenes	0.09	—	—	—	—	—	0.16	—	—	—	—	—
p-Cymene	0.09	—	—	—	—	—	0.16	—	—	—	—	—
Halogen derivatives:												
Chlorobenzene	0.11	—	—	—	—	—	0.2	—	—	—	—	—
Bromobenzene	0.09	—	—	—	—	—	0.12	—	—	—	—	—
Iodobenzene	0.11	—	—	—	—	—	0.18	—	—	—	—	—
o-Dichlorobenzene	0.11	—	—	—	—	—	0.24	—	—	—	—	—
p-Dichlorobenzene	0.12	—	—	—	—	—	0.37	—	—	—	—	—
2:3:5:6-Tetrachloronitrobenzene	17.5	—	—	—	—	—	*	—	—	—	—	—
Cyclohexyl bromide	0.16	—	—	—	—	—	0.32	—	—	—	—	—
Mercury compounds:												
Phenyl mercuri-acetate	0.01	—	—	—	—	—	0.12	—	—	—	—	—
An organic mercurial seed dressing containing 1% mercury	0.8	—	—	—	—	—	3.75	—	—	—	—	—
Amino-compounds:												
Aniline	0.04	—	—	0.06	0.05	0.03	0.12	—	—	0.13	0.13	0.13
p-Aminophenyl arsonic acid	1.25	—	—	0.6	—	—	2.5	—	—	*	—	—
Diphenylamine	12.5	—	—	—	—	*	*	—	—	—	—	*
Diphenylamine chloroarsine	10.0	—	—	—	—	—	20.0	—	—	—	—	—
Azo-compounds:												
Azobenzene	1.25	—	—	—	—	—	2.5	—	—	—	—	—
Amino-azobenzene-hydrochloride	10.0	—	—	—	—	—	*	—	—	—	—	—
Phenols:												
Phenol	0.4	0.3	0.3	0.2	0.11	0.11	1.3	1.9	0.5	0.3	0.3	*
40% low boiling phenols in emulsion	0.8	1.0	0.4	0.3	0.3	—	2.6	5.1	0.9	1.0	*	—
40% high boiling phenols in emulsion	5.0	2.5	1.2	0.5	—	—	12.5	5.0	1.5	1.2	—	—
60% high boiling phenolic bodies in emulsion	18.75	3.75	1.8	0.75	—	—	31.0	10.0	2.4	*	—	—
Sodium 2:4:5-trichlorophenate	0.5	2.0	0.9	0.1	0.06	—	1.25	4.3	1.6	0.5	*	—
o-Iodo-phenol	—	—	—	0.3	0.1	—	—	—	—	0.5	0.3	—
o-Nitro-phenol	0.25	—	—	—	—	—	0.25	—	—	—	—	—
p-Nitro-phenol	1.25	—	—	0.13	0.1	—	5.0	—	—	0.6	0.25	—
Sodium *o*-phenyl phenate	5.0	5.25	0.5	0.25	0.1	—	10.0	6.6	3.0	0.6	0.25	—
Sodium *m*-chloro-*p*-phenyl-phenate	10.0	7.2	1.5	0.25	0.13	—	17.5	14.3	*	0.6	*	—
2-Cyclohexyl cyclohexanol+2-phenyl cyclohexanol	4.7	—	—	—	—	—	7.1	—	—	—	—	—
50% cresol	3.7	1.0	0.9	0.4	0.2	—	7.5	2.5	1.5	0.6	*	—
Monochloroxylenols with some dichloroxylenols	0.5	—	—	—	—	—	3.8	—	—	—	—	—
2:4-Dichloro-*sym.*-xylenol	1.9	—	—	—	—	—	20.0	—	—	—	—	—
"Dettol" (halogenated xylenol in aromatic oils)	1.0	1.25	0.6	0.5	—	—	2.5	3.3	1.8	1.0	—	—
Thymol	1.0	—	—	—	—	—	1.9	—	—	—	—	—
Resorcinol	0.25	1.0	0.6	0.13	0.08	0.09	1.9	5.0	1.5	1.0	*	*
4-*n*-Hexylresorcinol	6.2	—	—	—	—	—	*	—	—	—	—	—
Ketone:												
p-Chloro-acetophenone	1.25	—	—	—	—	—	2.5	—	—	—	—	—

TABLE II—*continued*

Chemical	Percentage of chemical to fresh faeces required to reduce the number of larvae by: 90% when applied — Dry or undiluted	90% — In very concentrated solutions	90% — In concentrated solutions	90% — In moderately concentrated solutions	90% — In moderately dilute solutions	90% — In dilute solutions	99.9% when applied — Dry or undiluted	99.9% — In very concentrated solutions	99.9% — In concentrated solutions	99.9% — In moderately concentrated solutions	99.9% — In moderately dilute solutions	99.9% — In dilute solutions
Monobasic acids and derivatives:												
Benzoic acid	17.5	—	—	—	*	—	*	—	—	—	*	—
Benzyl benzoate	28.0	—	—	—	—	—	* (70.0)	—	—	—	—	—
Chloromethylphenoxy-acetic acid	18.7	—	*	*	—	—	31.0	—	*	*	—	—
1% trichlorophenylmethyliodosalicylate	19.0	8.3	7.0	—	—	—	25.0	16.6	*	—	—	—
Ester of dibasic acid:												
Dimethyl phthalate	11.9	—	—	—	—	—	45.0	—	—	—	—	—
Triphenylmethane dyes:												
Malachite green	3.75	—	—	1.0	—	—	12.5	—	—	*	—	—
Brilliant green	7.5	—	3.1	1.25	—	—	*	—	*	*	—	—
Magenta	50.0	—	*	*	—	—	*	—	*	*	—	—
Methyl violet	30.0	—	—	*	—	—	*	—	—	*	—	—
Gentian violet	35.0	—	3.1	1.25	—	—	*	—	*	*	—	—
Methyl green	7.5	—	2.5	1.0	—	—	17.5	—	*	*	—	—
Terpenes:												
Turpentine	0.55	—	—	—	—	—	1.3	—	—	—	—	—
Essential oil ex *Artemisia maritima* containing 65% β-thujone and 16% 1:8-cineole	0.1	—	—	—	—	—	0.17	—	—	—	—	—
10% of essential oil of *Artemisia maritima* in an aqueous alcoholic soap basis	0.74	0.75	0.75	0.5	*	—	0.91	1.1	1.3	1.1	*	—
β-Thujone	0.092	—	—	—	—	—	0.092	—	—	—	—	—
1:8-Cineole	0.23	—	—	—	—	—	0.58	—	—	—	—	—
Oil of chenopodium	0.1	—	—	—	—	—	0.25	—	—	—	—	—
5% oil of chenopodium with 95% castor oil	2.5	—	—	—	—	—	6.2	—	—	—	—	
Naphthalene and derivative:												
Naphthalene	0.19	—	—	—	—	—	0.38	—	—	—	—	—
β-Naphthol	12.5	—	—	—	—	—	*	—	—	—	—	—
Saponins:												
Saponin mixture	20.0	25.0	6.0	—	—	—	*	*	*	—	—	—
Heterocyclic Compounds												
Benzthiazole:												
2-Mercaptobenzthiazole	35.0	—	—	—	—	—	*	—	—	—	—	—
Pyridine and derivatives:												
Pyridine	0.19	0.16	0.18	0.19	0.19	—	0.25	0.25	0.24	0.25	0.25	—
α-Picoline	0.09	0.07	0.085	0.07	0.09	0.1	0.17	0.12	0.17	0.14	0.12	*
β-Picoline	0.18	0.12	0.11	0.14	0.15	—	0.18	0.16	0.17	0.23	0.23	—
γ-Picoline	0.18	0.12	0.11	0.14	0.12	—	0.18	0.16	0.17	0.23	0.24	—
2:6-Lutidine	0.09	0.07	0.05	0.07	0.08	0.08	0.17	0.12	0.08	0.09	0.12	*
32.9% β-Picoline+16.9% γ-picoline+50.2% 2:6-lutidine	0.23	0.12	0.1	0.12	0.12	—	0.23	0.15	0.13	0.23	0.15	—
2:4-Lutidine+2:5-lutidine	0.1	—	0.065	0.07	0.06	0.06	0.18	—	0.12	0.14	0.1	0.1
2:4:6-Collidine	0.17	—	—	0.11	0.06	0.09	0.23	—	—	0.17	0.12	*
3:5-Lutidine+2:3:6-collidine+2:4:6-collidine	0.12	—	—	0.09	0.05	0.05	0.24	—	—	0.19	0.15	0.08
2:4:5-Collidine+*o*-toluidine+*p*-toluidine	0.16	—	—	0.11	0.06	0.05	0.34	—	—	0.17	0.11	0.1
Quinoline derivatives:												
Quinoline	0.54	—	0.52	0.27	0.09	0.11	6.9	—	1.95	0.7	0.18	*
8-Hydroxyquinoline sulphate	0.19	0.7	0.3	0.2	0.06	0.04	0.5	1.3	0.9	0.3	0.1	0.07
Acridine derivative:												
Acriflavine	2.5	—	2.5	—	—	—	6.25	—	5.6	—	—	—

TABLE II—*continued*

Chemical	Percentage of chemical to fresh faeces required to reduce the number of larvae by											
	90% when applied						99.9% when applied					
	Dry or undiluted	In very concentrated solutions	In concentrated solutions	In moderately concentrated solutions	In moderately dilute solutions	In dilute solutions	Dry or undiluted	In very concentrated solutions	In concentrated solutions	In moderately concentrated solutions	In moderately dilute solutions	In dilute solutions
Benzthiazine derivatives:												
3-Keto-2 : 3-dihydro-benz-1 : 4-thiazine	5.0	—	—	0·5	—	—	25.0	—	—	*	—	—
3-Keto-2 : 3-dihydro-6 : 7-dimethoxy-benz-1 : 4-thiazine	50.0	—	—	*	—	—	*	—	—	*	—	—
Hydrochloride of 6-amino 3-keto-2 : 3-dihydro-benz-1 : 4-thiazine	7.5	—	—	*	—	—	25.0	—	—	*	—	—
Phenoxthine:												
Phenoxthine	1.25	—	—	—	—	*	*	—	—	—	—	*
Phenothiazine and derivatives:												
Phenothiazine	50.0	—	—	—	—	0.04	*	—	—	—	—	*
Mercurated phenothiazine	0.019	—	—	0.06	0.04	0.05	0.38	—	—	0.6	*	*
3 : 7-Dinitro-10-acetyl-phenothiazine-5-sulphoxide	50.0	—	*	—	—	—	*	—	*	—	—	—
Phenothiazine sulphoxide	*	—	—	0.4	—	—	*	—	—	*	—	—
Thionol hydrochloride	35.0	—	—	—	—	*	40.0	—	—	—	—	*
Phenothiazone	40.0	—	—	0.5	0.31	0.05	*	—	—	*	*	*
Lauth's violet	3.75	—	—	*	—	—	12.5	—	—	*	—	—
Lauth's violet $\equiv$ (phenol)$_2$	2.5	—	—	*	—	—	5.0	—	—	*	—	—
Lauth's violet $\equiv$ (β-naphthol)$_2$	5.0	—	—	*	—	—	*	—	—	*	—	—
Lauth's violet $\equiv$ (salicylic acid)$_2$	17.5	—	—	*	—	—	35.0	—	—	*	—	—
Lauth's violet $\equiv$ (2-hydroxy-3-naphthoic acid)$_2$	30.0	—	—	*	—	—	*	—	—	*	—	—
Lauth's violet $\equiv$ (1-phenyl-3-methyl-5-pyrazolone)$_2$	50.0	—	—	*	—	—	*	—	—	*	—	—
Brilliant alizarin	25.0	—	*	—	—	—	*	—	*	—	—	—
Methylene blue	50.0	12.0	2.5	—	—	—	*	*	*	—	—	—
Alkaloids:												
Amphetamine sulphate	—	—	—	0.38	0.13	—	—	—	0.75	*	—	—
40% nicotine sulphate	5.7	1.7	0.4	0.28	0.28	—	11.4	7.2	2.7	*	*	—
Tobacco dust	10.0	—	—	—	—	—	*	—	—	—	—	—
Arecoline hydrobromide	0.25	—	—	0.06	—	—	1.25	—	—	0.25	—	—
Quinine acid hydrochloride	6.25	—	*	*	—	—	17.5	—	*	*	—	—
Emetine hydrochloride	2.5	—	—	0.4	—	—	*	—	—	*	—	—
							(10.0)					
Plant product:												
Crushed flax seed	30.0	—	—	—	—	—	62.5	—	—	—	—	—
Urines and manure:												
Fresh poultry manure	40.0	—	—	—	—	—	50.0	—	—	—	—	—
Fresh horse urine	19.0	17.0	—	—	—	—	25.0	31.0	—	—	—	—
Fresh cow urine	37.5	—	—	—	—	—	60.0	—	—	—	—	—
Fresh human urine	50.0	*	—	—	—	—	*	*	—	—	—	—
Fresh dog urine	12.5	9.4	—	—	—	—	50.0	12.5	—	—	—	—

undiluted, or in strong or weak solutions. Frequently there were considerable differences in the minimum percentages of chemical required to produce this phenomenon even between solutions of the concentrated group or between solutions of the dilute group. The minimum percentages of chemical which had this effect, when applied as a solid or undiluted, or in concentrated or in dilute solutions, are shown in Table IV. Some chemicals had this effect in all or practically all the cultures; other chemicals only had this effect in some of the cultures; when this effect occurred fairly regularly the percentages in Table IV have been printed in italic type. Undoubtedly the list would have been longer if many of the chemicals had not already killed the larvae in the earlier stages. Many of these chemicals

TABLE III

The chemicals are listed which in 1 : 2,000 or in weaker solutions reduced the numbers of larvae by 90 per cent and 99.9 per cent; the percentages of chemical required to do this are also given

Chemical	Percentage of chemical to fresh faeces required to reduce the number of larvae by			
	90% when applied		99.9% when applied	
	In very dilute solutions	In extremely dilute solutions	In very dilute solutions	In extremely dilute solutions
Sodium iodide	0.018	—	*	—
Potassium iodide	0.016	—	*	—
Potassium iodate	0.025	—	*	—
Zinc iodide	0.016	—	*	—
Mercuric chloride	0.01	—	0.017	—
Ethoxy-ethyl-mercuric chloride	0.008	—	0.017	—
Iodine	0.02	—	*	—
Methyl iodide	0.003	0.002	0.009	0.007
Ethyl iodide	0.015	—	0.04	—
n-Propyl iodide	0.044	—	*	—
Methylene iodide	0.005	0.005	0.02	0.02
Iodoform	—	0.006	—	*
Allyl isothiocyanate	0.001	0.003	0.002	*
Phenothiazine	0.03	—	*	—

might not have killed the third stage larvae if they had not been able to damage the developing larvae.

Dilute solutions of sodium 2: 4: 5-trichlorophenate were the only dilute solutions which sometimes allowed some of the larvae to develop to the third stage before all the larvae died; the minimum concentration of this compound which did this was 0.3 g./100 c.c.

A few chemicals kill the eggs. Although this technique does not lend itself to the recovery of dead eggs, they were seen in a few of the cultures treated with potassium cyanate, 25 per cent (w/v) aqueous dimethylamine, diphenylamine, high boiling phenols, sodium 2: 4: 5-trichlorophenate, *o*-nitrophenol, sodium *o*-phenyl phenate, and sodium *m*-chloro-*p*-phenyl phenate. Less frequently dead eggs were seen in a few of the cultures treated with thiocyanate mixtures, a few of the halogen derivatives of paraffins, ethers, phenols and pyridine derivatives, and still less frequently a few eggs were seen in cultures treated with other chemicals.

A few chemicals occasionally make some of the larvae exsheath after they have reached the third stage; this was most noticeable with sodium hydroxide, sodium bicarbonate, aqueous sodium hypochlorite (1.2 per cent available chlorine), potassium hydroxide, and potassium permanganate.

Some chemicals stain the larvae and occasionally the stained larvae remain active. Chemicals which most frequently stain or darken larvae include calcium chloroacetate, ferric chloride, aminoazobenzene hydrochloride, malachite green, brilliant green, magenta, methyl violet, gentian violet, methyl green, acriflavine, phenoxthine, phenothiazone, Lauth's violet–(β-naphthol)$_2$, and methylene blue.

Occasionally *Trichostrongylus axei* larvae have been seen in these cultures; they and some of the larger larvae seem to be more resistant to some chemicals than are the smaller species of Sclerostomes.

Many chemicals and plant products have been tested which have little or no lethal effect on either the eggs or the free-living larvae. Dibutyl phthalate at 65 per cent, crushed sorgeum grass at 62.5 per cent, crushed sudan grass at 62.5 per cent, crushed tulip tree leaves at 62.5 per cent, Velsicol 1068 at 60 per cent did not produce a 90 per cent reduction in the number of larvae. The addition of 62.5 per cent of

TABLE IV

The chemicals which often allow some of the larvae to develop and then kill some or all of them are listed, with the minimum percentages of chemical which did this when applied as a solid or undiluted and in concentrated and dilute solutions. When there were dead third stage larvae in all or practically all the cultures the percentages are printed in italic type

Chemical	Minimum percentages of chemical which sometimes allow larvae to develop and then kill				
	Some			All	
	Chemical as a solid or undiluted	Chemical in concentrated solutions	Chemical in dilute solutions	Chemical as a solid or undiluted	Chemical in concentrated solutions
Sodium hydroxide	0.8	0.9	—	—	—
Sodium bicarbonate	7.5	—	—	—	—
Sodium iodide	—	0.04	0.03	—	—
Sodium tetraborate	0.63	—	—	—	—
Sodium silicofluoride	*0.25*	0.1	0.1	*0.8*	—
Potassium hydroxide	0.8	1.2	—	—	—
Potassium chlorate	5.0	1.0	—	—	—
Potassium permanganate	*1.9*	—	—	15.0	—
Ammonium thiocyanate	*0.25*	*0.2*	*0.2*	—	—
Cuprous chloride	*0.8*	—	—	*5.0*	—
Cupric chloride	*1.9*	*0.8*	—	—	1.25
Cupric sulphate	*1.9*	*1.0*	—	—	—
Cupric nitrate	*1.9*	*0.5*	—	*2.5*	*2.2*
Magnesium borate	0.38	—	—	—	—
Calcium oxide	20.0	—	—	50.0	—
Calcium hydroxide	50.0	—	—	—	—
Calurea	1.2	1.1	—	—	—
16% superphosphate	20.0	—	—	—	—
Calcium arsenate	*1.25*	—	—	—	—
Calcium chloroacetate	1.25	0.6	—	—	—
Zinc chloride	*1.25*	*0.5*	—	—	2.6
Zinc sulphate	*1.9*	*0.6*	—	—	—
Zinc sulphide	30.0	—	—	—	—
Ethyl-mercuric chloride	0.008	—	—	—	—
Ethoxy-ethyl-mercuric chloride	*0.02*	—	0.02	0.25	—
Mercuric iodide	*0.05*	—	—	1.0	—
Ferrous chloride	—	1.4	—	—	2.9
Ferrous sulphate	1.9	2.4	—	—	—
Ferric sulphate	2.5	—	—	—	—
Nickel chloride	2.5	1.7	0.1	—	—
Iodoform	0.05	—	—	—	—
Dichlorodiethyl ether	0.06	0.06	0.04	—	—
Tetramethylthiuram monosulphide	0.6	0.25	0.16	*3.75*	—
46% tetramethylthiuram disulphide	5.0	0.4	0.1	—	—
Hexamethylene tetramine	3.75	1.8	—	—	—
50% mixed thiocyanates	0.46	—	—	—	—
Hexaethyl tetraphosphate	*2.5*	—	—	—	—
Di-ethyl-*p*-nitrophenyl-thiophosphate	*0.32*	—	—	*4.7*	—
Sodium alkyl sulphates (Teepol X)	*5.25*	1.0	—	52.5	—
25% dimethylamine	2.3	1.7	—	—	—
Cetyltrimethylammonium bromide	*3.75*	1.1	—	—	—
20% solution of alkylammonium bromides	—	2.4	—	—	—
80% zinc dimethyl dithiocarbamate	*0.5*	0.4	0.2	—	—
80% ferric dimethyl dithiocarbamate	*0.6*	0.25	0.2	—	—
Guanidine hydrochloride	—	0.3	—	—	—
Methylguanidine sulphate	—	0.5	—	—	—
as-Dimethylguanidine sulphate	—	0.25	—	—	—
Diphenylguanidine	—	0.37	—	—	—
Creatine	—	0.5	—	—	—
Creatinine	—	0.9	—	—	1.85
Thiourea	0.19	0.1	0.06	—	—
Allantoin	1.0	—	—	*1.25*	—
Xanthine	*1.25*	—	—	10.0	—
Guanine	*1.0*	—	—	—	—
2:3:5:6-Tetrachloronitrobenzene	*1.9*	—	—	—	—
Benzene hexachloride	3.75	—	—	—	—
Phenyl mercuri-acetate	0.025	—	—	—	—
An organic mercurial seed dressing, containing 1% mercury	0.82	—	—	2.5	—
Aniline	0.01	—	—	—	—
Diphenylamine	*0.25*	—	—	—	—
Diphenylamine chloroarsine	6.25	—	—	—	—
Azobenzene	*0.6*	—	—	—	—
Aminoazobenzene hydrochloride	*0.6*	—	—	—	—
40% high boiling phenols in emulsion	5.0	2.0	—	—	—
Sodium 2:4:5-trichlorophenate	*0.12*	*0.03*	*0.03*	*0.8*	*0.6*
o-Iodophenol	—	0.19	0.19	—	—
p-Nitrophenol	*0.25*	0.12	—	*1.25*	—

TABLE IV—*continued*

Chemical	Minimum percentages of chemical which sometimes allow larvae to develop and then kill				
	Some			All	
	Chemical as a solid or undiluted	Chemical in concentrated solutions	Chemical in dilute solutions	Chemical as a solid or undiluted	Chemical in concentrated solutions
Sodium-*o*-phenyl phenate	*1.9*	0.3	—	*7.5*	4.1
Sodium *m*-chloro-*p*-phenyl phenate	*1.25*	*0.3*	0.2	*12.5*	*6.0*
2-Cyclohexyl cyclohexanol + 2-phenyl cyclohexanol	*1.8*	—	—	—	—
50% cresol	5.0	—	—	6.25	—
Monochloroxylenols with some dichloroxylenols	*0.2*	—	—	1.9	—
2: 4-Dichloro-sym.-xylenol	*0.5*	—	—	*2.5*	—
4-Hexyl resorcinol	*2.5*	—	—	*7.5*	—
3: 4-Methylenedioxy-phenyl-*n*-propyl-benzyl sulphide	2.5	—	—	—	—
p-Chloroacetophenone	*0.5*	—	—	1.25	—
Benzoic acid	*5.0*	—	—	*30.0*	—
Benzyl benzoate	*4.0*	—	—	—	—
Dimethyl phthalate	*4.5*	—	—	—	—
Malachite green	*5.0*	—	—	—	—
Brilliant green	*3.75*	—	—	—	—
Methyl violet	*7.5*	—	—	—	—
Gentian violet	*1.9*	3.1	—	—	—
Methyl green	*5.0*	—	—	—	—
β-Naphthol	*1.25*	—	—	*5.0*	—
2-Mercapto-benzthiazole	*2.5*	—	—	—	—
Quinoline	*0.27*	—	—	2.7	—
8-Hydroxyquinoline sulphate	0.19	*0.2*	0.1	—	0.8
6-Amino-3-keto-2: 3-dihydrobenz-1: 4-thiazine hydrochloride	5.0	—	—	—	—
Phenoxthine	*0.25*	—	0.03	—	—
Phenothiazone	2.5	0.5	0.06	—	—
Lauth's violet = ⅓(β-naphthol)$_2$	*3.75*	0.4	—	—	—
Lauth's violet = ⅓(2-hydroxy-3-naphthoic acid)$_2$	12.5	—	—	—	—
Methylene blue	30.0	—	—	—	—
Crushed sorgeum grass	50.0	—	—	—	—
Crushed sudan grass	62.5	—	—	—	—
Crushed flax seed	20.0	—	—	—	—
Fresh poultry manure	*20.0*	—	—	40.0	—

calcium oxide, of calcium hydroxide, of ground limestone, of basic slag, or of calcium phosphate (as rock phosphate) did not cause a 90 per cent reduction in the numbers of the larvae; but 20 per cent, and sometimes less, of calcium oxide and 50 per cent of calcium hydroxide killed some of the larvae after they had reached the infective stage, and 50 per cent of the former killed them all.

A 90 per cent reduction in the number of larvae was not caused by the addition to 40 g. of fresh horse faeces of 20 g. of the following substances: sublimed sulphur, ferrous sulphide, ferric oxide, cystine (but sometimes 15 per cent, or more, killed some larvae after they had developed), benzene hexachloride and its *alpha*, *beta*, *gamma*, *and delta* isomers separately (however, in some cultures treated with benzene hexachloride many of the larvae died after they had developed to the third stage), 1: 1: 1-trichloro-2: 2-di-*p*-chlorophenylethane (DDT), (the addition to a 44.3 per cent naphtha emulsion of 12.66 per cent of DDT, of DDD, of benzene hexachloride, or of its four isomers did not increase the lethal values of the emulsion), salicylanilide, santonin, derris powder, 6-amino-3-keto-2: 3-dihydro-benz-1: 4-thiazine, 6-chloro-3-keto-2: 3-dihydro-benz-1: 4-thiazine, 10-acetyl-phenothiazine, thionol, powdered areca nut, white hellebore powder, powdered cousso, pyrethrum powder, mowrah meal, and yeast; however, a few of these substances did kill some of the larvae after they had developed. Although 50 per cent of *m*-methoxy-*p*-hydroxy-benzaldehyde (vanillin) did not produce a 90 per cent reduction in the numbers of larvae, even small quantities caused some reduction.

The addition of 37 per cent of a 25 per cent emulsion of Filix mas reduced the number of larvae by 90 per cent, but even the addition of nearly twice as much did not give a 99.9 per cent kill; therefore much of the reduction in numbers may have been caused by the alteration in the physical condition of the cultures.

Neither 12.5 per cent of phenylethyl-*n*-octyl sulphide, of *p*-methoxyphenyl*iso*-propyl-*n*-octyl sulphide, of phenylethylbenzyl sulphide, nor of 3: 4-methylenedioxy-phenyl-*n*-propylbenzyl sulphide reduced the number of larvae by 90 per cent.

Extract of quassia was only tested in a concentrated solution, in which the maximum amount applied was only 6.9 per cent of the faeces; this quantity reduced the number of larvae by about 60 per cent, a reduction which could be explained by the altered physical condition of the cultures.

The addition of 5.0 per cent of " sulphoacetamide soluble " did not produce a 90 per cent reduction of larvae, neither were 1: 20 nor 1: 50 aqueous solutions effective.

Lauth's violet : (chromotropic acid)$_2$ as a 1: 100 aqueous solution had little effect on the numbers of larvae.

Neither the sodium salt of penicillin, containing 100,000 units, of which over 90 per cent were penicillin G, nor an atmosphere saturated with carbon monoxide, reduced the numbers of the larvae to any appreciable extent.

Discussion

Where the difference between the amount required to kill 90 per cent and 99.9 per cent is large, it can usually be concluded that the chemical must be in intimate contact with the eggs or larvae in order to kill them. When the chemical, in contact with horse faeces, produces a lethal gas, the quantity that kills 90 per cent may also kill 99.9 per cent. However, one culture with an unusually high count of larvae can reduce the apparent lethal value of a chemical, and therefore any unexpected order of potency should, if possible, be considered in comparison with the same chemicals in a different state.

In this technique the chemicals are in the presence of a comparatively large quantity of organic matter; therefore the chemical may occasionally have been altered before the larvae hatched from the eggs. Furthermore at 25° C. a few chemicals may volatilize before the eggs have made much development.

With most compounds, whether applied solid or undiluted or in solutions, there is generally a fairly close parallelism in the order of potency for a 90 per cent and for a 99.9 per cent kill. Brief notes on the many types of chemical tested are given below.

Inorganic compounds

Data for these chemicals are given in Table I. The majority of the metallic compounds tested were more effective in dilute solutions, both for a 90 and a 99.9 per cent kill, than in more concentrated solutions or in the solid state, probably because most of these chemicals have to be in intimate contact with the eggs or larvae in order to kill them. The majority of these compounds are, to some degree, larvicides. The metallic iodides and mercury compounds generally were the most potent, but most of the sulphates had low lethal values. The chlorides and nitrates had only moderate larvicidal activity. The same amounts of sodium, potassium, cupric, and calcium nitrates, applied as solids, were required to produce a 90 per cent kill, viz. 3.7 per cent. With the exception of ferrous

and ferric chlorides, approximately twice the amount of solid metallic chlorides were required to produce a 99.9 per cent kill as were required for a 90 per cent kill.

Sodium compounds.—The most effective of the solid sodium compounds was sodium iodide; the next most effective was sodium fluoride. In solution sodium iodide was again the most effective, but sodium silicofluoride was comparable with sodium fluoride. Sodium tetraborate, both as a solid and in solutions, produced a 90 per cent kill. The low lethal value of sodium hydroxide was surprising, considering that it has been recommended as a disinfectant against hookworm larvae; however, it is one of the chemicals which sometimes makes third stage larvae exsheath and which sometimes kills them after they have developed. Sodium chloride, which has frequently been used against hookworm larvae, is only a moderately effective larvicide. Sodium bicarbonate and sodium hypochlorite both made some of the third stage larvae exsheath.

Solid sodium silicofluoride in practically all the cultures allowed some of the larvae to develop to the third stage before killing them; this characteristic was less marked when this chemical was added in solution. Sodium tetraborate applied as a solid in some cultures allowed the larvae to develop before they were killed, but only in a very few cultures did this occur when solutions of this chemical were applied. Solid sodium chlorate was four times as potent as solid potassium chlorate for a 90 per cent kill, and nearly six times for a 99.9 per cent kill.

Potassium compounds.—Of the solid potassium compounds, potassium iodide was the most lethal, and although it was about as effective as sodium iodide in producing a 90 per cent kill, it was more potent in producing a 99.9 per cent kill; in solutions these two compounds were comparable to each other. In the concentrations tested, potassium iodide and iodate were about equal and were the most lethal of the potassium compounds examined. Potassium iodate was 300 times as effective as potassium chlorate for 90 per cent and 56 times for 99.9 per cent kills; again indicating the possible effect of the iodine atom in the molecule.

Potassium chlorate appeared to be much less effective than sodium chlorate, and potassium hydroxide was slightly less effective than sodium hydroxide. Potassium hydroxide was like sodium hydroxide in that it sometimes made the larvae exsheath after the second ecdysis. Especially when applied as a solid, it sometimes killed the larvae after they had reached the third stage.

" Muriate of potash " (two grades) and potassium nitrate, both of which are used as fertilizers, gave similar results as larvicides. Kainite which was slightly more effective, and is also used as a potash fertilizer, is included in the magnesium compounds. Potassium sulphate, also a fertilizer, was rather less effective. Applied as a solid, potassium permanganate killed many of the larvae after they had reached the third stage and made some of the others exsheath, but it is a weak larvicide.

Ammonium compounds.—Included in the ammonium compounds are some nitrogenous fertilizers. All the ammonium compounds gave a 99.9 per cent kill when 10 per cent, or less, was added to the cultures. Ammonium iodide was the most effective, whether applied dry or in solution. It was found that to produce a 99.9 per cent kill ammonium sulphate, applied dry, was twice as lethal as sodium sulphate and three times more effective than potassium sulphate. When ammonium thiocyanate was applied either as a solid or in solutions in many of the cultures some of the larvae died after reaching the third stage. This happened in a few of the cultures treated with ammonium sulphamate, but fewer larvae died.

Copper compounds.—Of the four copper compounds tested copper sulphate, which is used as an anthelmintic, was the least effective. Cupric chloride was almost twice as lethal as the cuprous salt in producing 90 and 99.9 per cent reductions in the numbers of larvae. These copper compounds, however, killed many of the larvae only after they had developed

to the infective stage (see Table IV); this occurred in the majority of the cultures to which these compounds were added in sufficient quantities.

Magnesium compounds.—Magnesium borate was the most effective of the few magnesium compounds tested; there was a tendency for this chemical to kill some of the larvae after they had developed.

Calcium compounds.—Most of the calcium compounds examined had little lethal value, although many farmers consider lime to be a larvicide, even on pastures. The heat of slaking is, of course, lethal, but otherwise only large quantities kill the larvae and then usually after they have developed.

As would be expected, calcium cyanide is very lethal, and was the most effective of the calcium compounds tested; there is sufficient moisture in faeces to make it release hydrogen cyanide. One per cent of calcium arsenate produced a 90 per cent reduction in the number of larvae, but 20 per cent was required to produce a 99.9 per cent reduction; however in most of the cultures treated with more than 1 per cent, only a few larvae developed and some of them died quickly. In some of the cultures treated with solid and very concentrated solutions of calcium chloroacetate some of the larvae died after they had reached the third stage.

Zinc compounds.—In common with the other metallic iodides which were tested, zinc iodide was the most effective of the zinc compounds, whether it was applied as a solid or in solution.

All four zinc compounds tested, including zinc iodide in very concentrated solutions, killed many of the larvae after they had reached the third stage. Zinc sulphide was very much less potent in reducing the numbers or in killing them after they had developed than zinc sulphate, which was one of the most effective sulphates.

Mercury compounds.—All the mercury compounds were lethal, especially ethyl-mercuric chloride. The larvicidal effect appeared to be depressed somewhat by the introduction of an ethoxyl group as was shown by the fact that ethyl-mercuric chloride was 15 times (for a 99.9 per cent kill) and 6 times (for a 90 per cent kill) as potent as ethoxyethyl-mercuric chloride, which had the same value as the unsubstituted mercuric chloride for a 90 per cent kill.

It is surprising that mercuric iodide was the least effective of the pure mercury compounds tested. This may be due to its insolubility. Levine (1951a) found that 0.011 per cent mercuric iodide produced a 99 per cent kill. We found that 2.5 per cent gave a 99 per cent reduction in the numbers of larvae, but that 1 per cent killed all the larvae which reached the infective stage, and considerably less killed most of them. In a few of the cultures treated with mercury compounds some of the larvae died after they had reached the third stage. In a few of the cultures treated with mercuric chloride, this chemical seemed to have had a selective lethal action as only a few of the larger strongyloid larvae were alive. Ethoxyethyl-mercuric chloride also showed some selective lethal activity.

Boron compounds.—Ortho-boric acid did not differ much in its lethal potency from sodium tetraborate and magnesium borate.

Carbon monoxide.—Sealing the jars containing the cultures is in itself lethal to developing larvae; it was therefore difficult to be sure that carbon monoxide remained in contact with the eggs and larvae for any length of time; however this gas did not appear to be lethal.

Sulphur.—There seemed to be a tendency for the numbers of larvae recovered from the cultures treated with sublimed sulphur to be greater than those from the controls. This can happen with a fungicide, which is not a larvicide, if there are fungi which parasitize larvae present in the cultures. In a few cultures to which nematocidal fungi were added the number of larvae was reduced by over 90 per cent in a few weeks.

Iodine.—Some iodine compounds have already been discussed; others will be in later sections (see Tables I and II). In the three samples which were tested, iodine in colloidal form lost some of its larvicidal potency.

Manganese compounds.—Neither manganous chloride nor manganous sulphate have a high larvicidal value. Applied in solid form the chloride was two or three times as effective as the sulphate; in solutions the differences were less. Manganous sulphate was one of the more effective sulphates. In a few of the cultures treated with both compounds some of the larvae died after they had developed to the third stage.

Iron compounds.—Ferrous iodide was the most potent of the iron compounds tested, but generally their larvicidal potency was low. Added to the cultures as solids, ferrous and ferric chlorides and ferrous and ferric sulphates were very similar in their effects. Some of the larvae in many of the cultures treated with the chlorides and sulphates of ferrous and ferric iron died after they had developed to the third stage, especially in the cultures treated with the ferrous compounds.

Cobalt and nickel compounds.—Cobalt and nickel chlorides were the only salts of these metals tested; they had somewhat similar lethal values. Nickel chloride had a greater tendency to kill the larvae after they had reached the third stage, but the numbers killed were never great, although quite small percentages killed a few.

Organic compounds (aliphatic)

The larvicidal values of the organic compounds are given in Table II.

Halogen derivatives of paraffins.—In this group are several chemicals which are used for the control of plant nematodes. Only a few of these compounds are appreciably soluble in water. Those which were tested in solution showed few significant differences from their values when applied undiluted. The most noteworthy feature of these derivatives is the lethal effect of the iodides (see Table III). The corresponding bromides were less lethal, but still had a considerable effect. The corresponding chlorides tested were found to be less effective than the bromides. Ethyl chloride (b.p. 12° C.) appeared to have a very low value, but it may have dispersed before some of the eggs had hatched.

Chloropicrin (nitrochloroform) affords an example of the apparent effect of a nitro-group. The substitution of a nitro-group for the hydrogen atom in chloroform seems to increase lethal activity 60 times for a 90 per cent kill and nearly 200 times for a 99.9 per cent kill. The amounts of chloropicrin required for 90 and 99.9 per cent kills were the same.

A comparison of carbon tetrachloride with carbon tetrabromide shows the latter compound to be nearly 70 times as effective for 90 per cent and almost 240 times for 99.9 per cent kills, again showing the influence of bromine in organic compounds. The results with carbon tetrabromide are sufficiently outstanding to justify further investigation into its nematocidal properties.

A comparison of the normal alkyl bromides is interesting. The percentage of chemical required to sterilize the faeces has been plotted against the number of carbon atoms in Fig. 1; the graphs for 90 and 99.9 per cent reductions are similar. The maximum quantity of chemical required was reached with *n*-amyl bromide followed by a sharp fall to *n*-hexyl bromide.

n-Butyl bromide was 12 times as powerful as *tert.*-butyl bromide for a 90 and 5 times for a 99.9 per cent kill.

A comparison of methylene dichloride, chloroform, and carbon tetrachloride shows that there is a decrease in potency with an increase in the number of chlorine atoms. In the corresponding bromo-compounds, the potency increases with the number of bromine atoms.

The iodine compounds are more effective than the corresponding bromine compounds, which, in turn, are more so than the corresponding chloro-derivatives (cf. alkyl halides).

Ethylidene dichloride is less effective than ethylene dichloride. DD, a mixture of 1 : 3-dichloropropylene, 1 : 2-dichloropropane and higher chlorides, had approximately the same larvicidal value whether applied undiluted or in the naphtha emulsion.

Iodoform was the only compound of this group which allowed some of the larvae to develop before they died; this happened whether the iodoform was mixed in the faeces or suspended above them; in the latter case larger quantities were required.

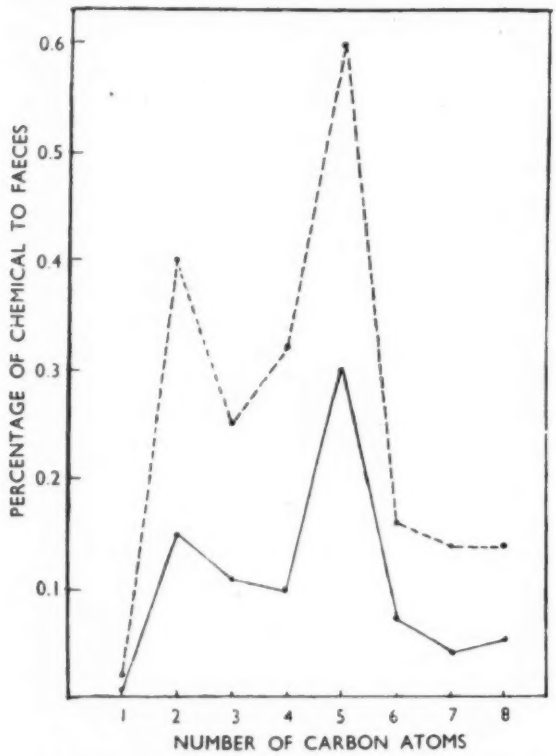

FIG. 1.—A graph to show the relationship between the number of carbon atoms in the *n*-alkyl bromides and the percentage of chemical required to produce a 90 per cent kill (continuous line) and a 99.9 per cent kill (broken line).

Halogen derivatives of ethylene.—An increase in the number of chlorine atoms results in increased potency in the chlorine derivatives of ethylene (cf. the chlorine derivatives of the paraffins). *Cis-* and *trans*-dichloroethylene were about equally effective in producing a 90 per cent kill, but the *cis*-isomer appeared to be slightly more powerful than the *trans*- for a 99.9 per cent kill. It may be that the proximity of the chlorine atoms to one another in the *cis*-compound is a contributory factor. The values for perchloroethylene were the same for 90 and 99.9 per cent kills.

Alcohols (*methyl alcohol series*).—It was found that effectiveness in producing a 90 per cent kill increased as the series of normal alcohols was ascended. *n-* and *iso*-propyl alcohols and *tert.*-butyl alcohol had the same value for a 90 per cent kill (1.5 per cent).

For a 99.9 per cent kill, methyl and ethyl alcohols were of equal effectiveness (5.0 per cent), and *n*-propyl, *n-* and *tert.*-butyl alcohols also had the same larvicidal values (1.5 per cent). The most interesting feature, however, was the greater larvicidal effect of *n*-propyl, *n*-butyl, and *tert.*-butyl alcohols, which were each twice as lethal as their *iso*-isomers. There were no significant differences whether these chemicals were applied undiluted or in aqueous solutions.

The addition of some compounds to the cultures occasionally produces third stage larvae which, although alive, are shrunken; a few of these larvae were found in cultures treated with methyl alcohol.

Ethers.—The two substituted diethyl ethers were more powerful larvicides than the unsubstituted compound. The substitution of two chlorine atoms for two hydrogen atoms increased the effect 30 times for a 90 per cent kill, and 36 times for a 99.9 per cent kill. In some cultures treated with dichlorodiethyl ether a few of the larvae died after they reached the third stage.

Sulphides.—Tetramethylthiuram monosulphide and the corresponding disulphide, both of which were applied in slight suspension, were very much more effective when added to the cultures in this way than when they were added dry. In most suspensions the disulphide, although 54 per cent of the mixture added was an inert dispersal agent, was more effective than the monosulphide in reducing the numbers of the larvae; but in the cultures treated with the monosulphide there was a greater tendency for many of the larvae to die after they had reached the third stage.

Formaldehyde derivative.—A few of the larvae in cultures to which hexamethylene tetramine had been added died after they had reached the third stage.

Esters.—Ethyl iodoacetate, like other lachrymators, has a fairly high larvicidal value. With the exception of this compound, the other esters which were tested all allowed some larvae to reach the third stage before killing them; this characteristic was very marked when hexaethyl tetraphosphate, diethyl-*p*-nitrophenylthiophosphate or Teepol X were added undiluted.

Amines.—In many of the cultures to which dimethylamine had been added as a 25 per cent aqueous solution dead eggs were found, and a few were found in four cultures to which weaker solutions had been added. When this compound was added to the cultures in small quantities some of the larvae reached the third stage before they were killed.

Cetyltrimethylammonium bromide, a wetting agent, and the 20 per cent solution of alkylammonium bromides, dissolved in methylated spirit, both killed some larvae slowly, so that they had reached the third stage before they died, but this occurred much more frequently with the former.

Allyl compounds.—The allyl compounds tested were very effective larvicides, particularly allyl isothiocyanate (a lachrymatory liquid) and allyl iodide, which were approximately equal. Allyl isothiocyanate and allyl alcohol, the two allyl compounds which were tested as aqueous solutions, showed very little difference in value whether applied undiluted or in solutions. When the allyl halides were compared, it was found, as with the alkyl halides, that allyl iodide was more powerful than the bromide, which in turn was more effective than the chloride. Compared with alcohols of the methyl alcohol series, allyl alcohol (a lachrymatory liquid) was much more potent.

Amino acid.—The addition of cystine to the cultures even in large proportions only reduced the number of larvae slightly, but a few more died after they had reached the third stage.

Carbonic acid derivatives.—Both zinc and ferric dimethyldithiocarbamates were much more effective applied in suspension than added dry. Small quantities of both compounds markedly reduced the numbers of larvae which reached the third stage and quite small quantities killed some of the larvae after they had developed; however, not even large quantities of the dry compounds prevented all the larvae developing to the third stage or killed all that did so.

The substitution of a sulphur atom for oxygen in urea increased the effect eight times for 90 per cent and twice for 99.9 per cent reductions in the number of larvae, when the

compounds were applied as solids. In solution, thiourea was generally more effective, and, in addition, many of the larvae in cultures treated with thiourea solutions died after they had developed to the third stage.

The frequency with which some larvae developed to the third stage and then died was marked in cultures treated with methylguanidine sulphate, diphenyl guanidine, and creatinine in the more concentrated solutions; this also occurred in the more concentrated solutions of guanidine hydrochloride, *as*-dimethylguanidine sulphate and creatine, but to a less marked extent.

Ureides and purine derivatives.—Although the addition of 50 per cent of allantoin in the dry state to the cultures did not reduce the number of third stage larvae found by more than about 99.7 per cent, the addition of only 1.25 per cent ensured that they had all died after they had developed to the third stage.

Both xanthine and guanine added to the cultures of fresh horse faeces as solids killed some of the third stage larvae.

Organic compounds (carbocyclic)

Hydrocarbons.—The xylenes (*ortho-*, *meta*, and *para*-mixed) and *p*-cymene were equally potent and very effective larvicides, more so than toluene, which was more effective than benzene. It would appear that an increase in the number of alkyl groups increases the larvicidal effect.

Halogen derivatives.—Chloro-, bromo-, and iodo-benzene, *o*- and *p*-dichloro-benzene, and cyclohexyl bromide were quite effective. Benzene hexachloride (containing a mixture of isomers) and its α-, β-, γ-, and δ-isomers separately, and DDT were all tested as solids, but had low or no larvicidal values. However, in many of the cultures to which 3.75 per cent or more of benzene hexachloride was added some of the larvae died after reaching the third stage, but even 50 per cent of benzene hexachloride only reduced the number of larvae which were extracted from the cultures by about 80 per cent.

Chloro-, bromo-, and iodo-benzenes were tested in very dilute solutions, but they did not affect the eggs or larvae.

Although the addition of 50 per cent of 2:3:5:6-tetrachloronitrobenzene to fresh horse faeces did not quite reduce the number of larvae recovered by 99.9 per cent, yet the addition of only 1.9 per cent ensured that some of the larvae died after reaching the third stage.

Mercury compounds.—The two mercuric compounds confirm the lethal effect of the mercury atom, which was noticed in the inorganic and aliphatic compounds of mercury. Both tended to kill some of the larvae after they had developed.

Amino-compounds.—Aniline is an effective larvicide, but the other compounds, which were tested, were not so effective in reducing the numbers of the larvae. However, diphenylamine in small quantities affected the viability of the larvae; for example, in some cultures treated with 0.5 per cent, and over, a considerable number of larvae were alive when they were extracted from the cultures, but ten days later all or practically all, had died. Diphenylamine tended to darken the larvae.

Azo-compounds.—In cultures treated with azobenzene and with aminoazobenzene hydrochloride some larvae died after they had reached the third stage. Aminoazobenzene hydrochloride makes the larvae sluggish; therefore it is probable that a lower proportion of live larvae than normal was recovered from the cultures. This compound occasionally stains live larvae.

Phenols.—The relative effectiveness of the phenols was increased by dilution with water. The low boiling-point phenols were more effective than the less volatile phenols.

Filix mas emulsion and 4-*n*-hexylresorcinol, two compounds with specific anthelmintic properties, have low larvicidal potencies. Apart from chemical reasons this apparent anomaly may be due to the physical condition of the Filix mas emulsion and to the poor solubility of 4-*n*-hexylresorcinol (1 : 2,000) in water, which would only allow it to penetrate the faeces slowly. There is, perhaps, confirmation of this in the fact that many larvae in cultures treated with 4-*n*-hexylresorcinol develop to the third stage before being killed. Resorcinol, on the other hand, is quite effective; so are phenol, sodium 2 : 4 : 5-trichlorophenate, *o*-nitro-phenol, and thymol.

The same amount of *o*-nitro-phenol is required for a 90 as for a 99.9 per cent kill. This compound is 5 times as effective for a 90 per cent and 20 times as effective for a 99.9 per cent kill as the *para*-isomer. Sodium 2 : 4 : 5-trichlorophenate has about the same effect as phenol, but the larvicidal effect is considerably reduced in sodium *o*-phenylphenate and still more so in sodium *m*-chloro-*p*-phenyl phenate. The introduction of a phenyl group may be the contributory factor.

Among the phenols were compounds which appeared to kill the eggs and many which allowed some of the larvae in the cultures to develop before killing them. These apparent contradictions may be due to the very local action of some of these compounds. For example, when the chemicals were mixed in the cultures they would immediately be in contact with some eggs; during development, more larvae would come in contact with the chemicals, but some might not do so until they had developed to the third stage.

Ketone.—*p*-Chloroacetophenone is another lachrymatory compound, but its comparatively low vapour pressure may account for the fact that its larvicidal potency is lower than that of the other lachrymators, and for the fact that some larvae in the cultures were able to develop to the third stage before being killed.

Monobasic acid.—Bloomfield (1949) found that benzoic acid was the only constituent of urine which killed eggs, preinfective and infective larvae. In the present investigation, the addition of 50 per cent of benzoic acid only reduced the number of larvae by 97 per cent; 30 per cent was the minimum percentage which killed all the larvae which reached the third stage in all the cultures although in some cultures all the larvae were killed by smaller quantities. This suggests that this compound must be in close contact to be lethal to eggs or larvae.

Esters of dibasic acids.—Dimethylphthalate had a very low lethal value, but it was more potent than the dibutyl ester, and killed many of the larvae which developed to the third stage, which dibutylphthalate did not do.

Triphenylmethane dyes.—Added as solids these dyes have a low potency as larvicides, although some of them are recognized anthelmintics against some nematodes which parasitize man. Gentian violet was much more effective in solution than in the solid state in producing a 90 per cent kill, but did not produce a 99.9 per cent kill. In some of the cultures treated with these dyes some of the third stage larvae, both live and dead, were stained; the staining was particularly frequent in the larvae from cultures treated with magenta, methyl violet, and gentian violet. As some of the larvae died fairly soon after reaching the third stage (see Table IV) it seems probable that these dyes have a slow lethal action on the free-living stages in faeces at 25° C.

Terpenes.—Turpentine, essential oil of *Artemisia maritima*, containing 65 per cent β-thujone and 16 per cent 1 : 8-cineole, and oil of chenopodium were effective larvicides, but santonin was of no value. This may be caused by its physical condition, although as a 1 : 500 aqueous solution 25 c.c. in 40 g. of fresh faeces only caused a 70 per cent reduction in the number of third stage larvae which were found; it is really a vermifuge.

1 : 8-Cineole and β-thujone were tested separately and found to be effective. β-Thujone had the same value for 90 and 99.9 per cent kills, and was 6 times more potent than 1 : 8-cineole for a 99.9 per cent kill.

Naphthalene and β-naphthol.—Judged by the numbers of larvae which developed to the third stage, the larvicidal effect of naphthalene was weakened very considerably by the introduction of the hydroxyl group in the β-position. This was rather unexpected as the introduction of a hydroxyl group sometimes increases anthelmintic activity. Many of the third stage larvae died in the cultures treated with β-naphthol, but even for this delayed killing effect considerably more β-naphthol was required than was necessary to produce a 99.9 per cent reduction in the numbers of larvae in the naphthalene treated cultures.

Organic compounds (heterocyclic)

Benzthiazole.—In practically all of the cultures treated with 2.5 per cent, or more, of 2-mercapto-benzthiazole some of the larvae which reached the third stage rapidly died, although 35 per cent was required to produce even a 90 per cent reduction in the number of larvae recovered from the cultures.

Pyridine derivatives.—Pyridine and derivatives were found to be effective larvicides and had values ranging from 0.09 to 0.23 per cent for 90 per cent and from 0.17 to 0.25 per cent for 99.9 per cent kills. The lethal values of these chemicals were little changed on dilution with water.

Quinoline and derivative.—In many of the cultures treated with undiluted quinoline and with concentrated solutions of 8-hydroxyquinoline sulphate many larvae died after they had reached the third stage; some larvae died in a few of the cultures treated with these two compounds in less concentrated solutions.

Acriflavine.—Acriflavine sometimes killed the larvae after they had reached the third stage, yet at other times the third stage larvae were deeply stained but were apparently unharmed.

Benzthiazine derivatives.—These derivatives had very little larvicidal value; the simplest compound, 3-keto-2 : 3-dihydrobenz-1 : 4-thiazine, was the most lethal. In the cultures treated with the aminohydrochloride many of the larvae died after they had reached the third stage; this compound adhered to their sheaths.

Phenoxthine.—Although the addition of very small quantities of phenoxthine to cultures reduced the numbers of larvae which reached the third stage very considerably, and many of the larvae which did reach that stage died, yet even 50 per cent of phenoxthine added to the cultures did not prevent some larvae developing. In twenty out of forty-five cultures treated with 2.5 per cent, or more, of this compound all the larvae were dead.

Phenothiazine and derivatives.—Mercurated phenothiazine was a powerful larvicide, but phenothiazine and the other phenothiazine derivatives applied as solids were not effective larvicides. Phenothiazine, phenothiazone, and methylene blue were more effective in producing a 90 per cent kill in solution or suspension than when applied dry, but none of these chemicals produced a 99.9 per cent kill. The fact that in each culture of horse faeces there are larvae of many species probably decreases the chance of a 99.9 per cent kill with any chemical which is a specific larvicide; as an anthelmintic phenothiazine is selective.

Lauth's violet was not particularly effective, but when coupled with phenol, its potency was slightly increased. Other couplings with Lauth's violet decreased its value; but many of the larvae died after they reached the third stage in cultures treated with Lauth's violet⇄(β-naphthol)$_2$, some in the cultures treated with Lauth's violet⇄(2-hydroxy-3-naphthoic acid)$_2$, with phenothiazone and with over 30 per cent of methylene blue.

Alkaloids.—Of the alkaloids added as solids to the cultures arecoline hydrobromide was the most effective. Nicotine sulphate (40 per cent solution) diluted twenty to fifty times with water was considerably more effective than in the more concentrated solution.

Plant products.—The plant products which were tested had very little larvicidal value. The crushed leaves were tried in order to determine whether or not they might slowly release sufficient hydrogen cyanide to be of value in destroying plant nematodes, if crops with high nitrogen contents were ploughed in as green manure.

Urines and manure.—Although fresh poultry manure and urines have a low larvicidal value, it has been found that they have a practical value for killing larvae in manure heaps and in night-soil.

Physical properties

In general there appeared to be no correlation between boiling-points and larvicidal effect. In the inorganic compounds there seemed to be a general tendency for larvicidal effect to increase with solubility in water, but there were exceptions. In the organic compounds no increase in larvicidal effect was observed with increase of solubility, when the chemicals are taken as a whole. It would appear that, unless the chemical has to be in intimate contact with the eggs or larvae, solubility in water is of secondary importance. No simple correlation exists between larvicidal action and molecular weight, vapour pressure or parachor.

Conclusions

Many chemicals were applied to fresh horse faeces in a confined space at a temperature of about 25° C.; from the results it has been possible to draw some conclusions on their effect as larvicides against the free-living stages of Sclerostomes.

1. Of the metallic compounds which were tested, those of mercury were the most potent larvicides.
2. The metallic iodides are generally the most potent of the inorganic salts; compounds of fluorine and boron are less effective larvicides.
3. The nitrates have comparable larvicidal values to the chlorides, both of which are more potent than the sulphates.
4. The ammonium compounds are larvicidal and have a practical value in killing larvae in night-soil and middens.
5. The copper compounds are notable for the slowness with which they kill Sclerostome larvae.
6. Calcium compounds, in spite of their reputation, are very weak larvicides.
7. Sulphur has no larvicidal action.
8. Most inorganic compounds are more effective when applied in solution than as solids.
9. Iodo-derivatives of the paraffins are very lethal, the order of potency of halogenated paraffins being iodo>bromo>chloro.
10. The substitution of a nitro-group for the hydrogen atom in chloroform considerably increases its larvicidal value.
11. The larvicidal value of carbon tetrabromide is high; this chemical should be tested against other helminths.
12. *n*-Amyl bromide is the least effective of the normal alkyl bromides in the series methyl to octyl.

13. *n*-Butyl bromide is more lethal than *tert.*-butyl bromide.

14. In the halogen derivatives of the paraffins there is a decrease in larvicidal potency with an increase in the number of chlorine atoms, but an increase in potency with an increase in the number of bromine atoms.

15. In the halogen derivatives of ethylene an increase in the number of chlorine atoms increases the larvicidal potency.

16. The effectiveness of the normal alcohols to produce a 90 per cent reduction in the number of larvae increases as the series of alcohols is ascended.

17. *n*-Propyl, *n*-butyl, and *tert.*-butyl alcohols are more lethal than their *iso*-isomers.

18. The substitution of two chlorine atoms for two hydrogen atoms in ether considerably increased the larvicidal value.

19. Tetramethylthiuram disulphide is more effective than the monosulphide in reducing the number of larvae, but both have to be in intimate contact with the larvae to kill them.

20. Dimethylamine and diphenylamine are both ovicidal as well as larvicidal, but the latter has a peculiar delayed larvicidal action.

21. Allyl compounds are very potent larvicides, especially the isothiocyanate and the iodide. With the halides, the order of potency is I>Br>Cl.

22. Both zinc and ferric dimethyldithiocarbamates have to be in intimate contact with larvae to kill them.

23. The substitution of a sulphur atom for oxygen in urea increases its larvicidal value considerably.

24. Many of the carbonic acid derivatives have a slow lethal action.

25. In the aromatic hydrocarbons an increase in the alkyl groups increases the larvicidal values.

26. Chloro, bromo- and iodobenzene, *o*- and *p*-dichlorobenzene are quite potent larvicides, but benzene hexachloride has a very low larvicidal value, and 2: 3: 5: 6-tetrachloronitrobenzene is a very slow killer.

27. Azobenzene and aminoazobenzene hydrochloride also seem to have a delayed lethal effect.

28. A number of phenolic compounds are effective larvicides, but several of them, including sodium 2: 4: 5-trichlorophenate, sodium-*o*-phenyl phenate, and sodium *m*-chloro-*p*-phenyl phenate, allow many of the larvae to develop to the third stage before they are killed. The introduction of a phenyl group reduces the larvicidal value of sodium 2: 4: 5-trichlorophenate. *o*-Nitro-phenol is much more effective than the *para*-isomer.

29. Some of the triphenylmethane dyestuffs also have a delayed lethal action.

30. The value of β-thujone should be tested against other helminths.

31. β-Naphthol is a less effective larvicide than naphthalene.

32. Pyridine and its derivatives are effective whether applied undiluted or as solutions.

33. As larvicides the benzthiazine derivatives have low values.

34. Phenoxthine has a slow lethal effect.

35. Phenothiazine and its derivatives, except mercurated phenothiazine, have low larvicidal values, but were more effective in solutions.

36. Urines and poultry manure, because of their availability on farms and their value as fertilizers, can be used in well-made middens to kill bursate nematode larvae, although their larvicidal potency is low.

37. The lachrymators, which were tested, have high larvicidal values.

38. Solubility is no guide to larvicidal effect; except in a number of inorganic compounds, where larvicidal effect increases with increase of solubility in water.

39. There is no correspondence between boiling-points, molecular weights, vapour pressures and parachors, and larvicidal values.

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THE GANGLION BLOCKING PROPERTIES OF HOMOLOGOUS COMPOUNDS IN THE METHONIUM SERIES

BY

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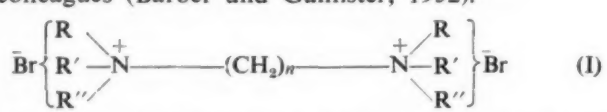
Speculation on the relationship of pharmacological activity to chemical structure is a fascinating occupation, though a complex one. An apparently small alteration in molecular configuration may profoundly change the actions of a compound ; a well-known example of this is the change in neuromuscular blocking activity brought about by the replacement of methyl by ethyl groups in quaternary ammonium ions (Ing, 1936). In a preceding paper (Wien and Mason, 1951) we found that the ability of hexamethonium to paralyse autonomic ganglia was influenced by the replacement of methyl by ethyl groups on the quaternary nitrogen atoms, and we have now extended our observations to a more systematic study of the tetra- to the hepta-methylene members in this alkamethonium series, as well as to several other quaternary ammonium compounds.

METHODS

The methods were the same as those described previously (Wien and Mason, 1951) for determining the paralysing effects of substances on transmission through the superior cervical ganglion of the cat ; on the peristaltic reflex of the isolated guinea-pig intestine ; and on transmission at the neuromuscular junction by the phrenic nerve-diaphragm preparation of the rabbit. Each result in Table I was the mean of several, at least two, experiments on each test object, permitting several comparisons, but no statistical analysis was attempted. In the experiments on the nictitating membrane care was taken to space the injections far enough apart (15 to 20 minutes) to prevent sub-threshold amounts of a previous injection affecting a later one ; the order of giving the compound to be tested and the standard for comparison was varied as a further safeguard. Toxicity estimations were determined intravenously in mice.

Compounds.—We examined sixteen polymethylene bis-trialkylammonium dibromides of general formula (I), where $n=4$, 5, 6, or 7, and $R=R'=R''=Me$, or $R=R'=Me$ and $R''=Et$, or $R=Me$ and $R'=R''=Et$, or $R=R'=R''=Et$. They were all prepared by our colleagues (Barber and Gaimster, 1952).



(I)

We also examined tetraethylammonium, triethylmethylammonium, diethyldimethylammonium, ethyltrimethylammonium, and tetramethylammonium bromides. The compound γ-phenoxypropyltriethylammonium iodide was kindly supplied by Professor

R. Paul, and some of its actions have been described by Depierre and Jacob (1950); it was also prepared by our colleague Dr. J. N. Ashley. The methyl homologue, γ-phenoxypropyltrimethylammonium bromide, has been described by Hey (1952) and it was kindly supplied by him.

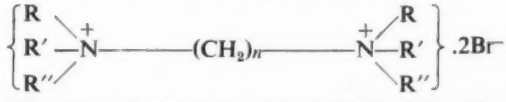
RESULTS

Ethyl homologues in the alkamethonium series

The parent bis-trimethylammonium compounds were described by Barlow and Ing (1948) and studied in some detail by Paton and Zaimis (1951), but the actions of the ethyl homologues have not hitherto been investigated. Barlow and Ing (1948) prepared the bis-triethylammonium series ($n=2$, 3, 4, 5, 7, 8, 9, 10, and 13), but did not study them in detail. Our results on this series of compounds are summarized in Table I.

TABLE I

THE EFFECT OF SUCCESSIVE REPLACEMENT OF METHYL BY ETHYL GROUPS ON BOTH QUATERNARY NITROGEN ATOMS IN THE FORMULA



Compound				Toxicity (mice)		Relative potency paralysing ganglia (hexamethonium = 100)		Neuromuscular paralysis (*d*-tubocurarine = 100)
n	R	R′	R″	LD50 mg./kg. i.v.	% limits of error (P = 0.05)	Sympathetic (Cat superior cervical)	Parasympathetic (Guinea-pig ileum)	Rabbit phrenic nerve-diaphragm
4	Me	Me	Me	122	(92–109)	1	5	Inactive
	Et	Me	Me	61	(89–113)	10	25	,,
	Et	Et	Me	35	(90–111)	100	25	,,
	Et	Et	Et	20	(91–110)	5	5	,,
5	Me	Me	Me	62	(81–119)	65	75	0.4
	Et	Me	Me	16	(84–120)	150	150	0.4
	Et	Et	Me	22	(91–110)	125	100	0.4
	Et	Et	Et	8	(85–118)	7	10	0.4
6	Me	Me	Me	50	(88–113)	100	100	0.5
	Et	Me	Me	22	(89–112)	150	200	0.2
	Et	Et	Me	12.5	(89–112)	75	100	0.4
	Et	Et	Et	2.5	(91–110)	<5	<2	4.0
7	Me	Me	Me	12	(90–112)	12	30	1.0
	Et	Me	Me	15	(94–107)	10	30	2.0
	Et	Et	Me	9	(91–111)	15	2	3.0
	Et	Et	Et	6	(91–111)	2	<2	5.0

These results showed several interesting facts. It was found that the substitution of ethyl for methyl groups profoundly affected the properties of the compounds. There was an increase in toxicity as more ethyl groups were introduced, reaching a maximum in the bis-triethylammonium compounds. This increase in toxicity was accompanied, in the hexa- and hepta-methylene members, by appreciable neuromuscular paralysis as well. In the whole animal, this neuromuscular paralysis may

be even more marked, as with decamethonium, which is several times more potent than *d*-tubocurarine in the sciatic-tibialis preparation of the cat, but less potent on the isolated phenic nerve-diaphragm preparation of the rabbit or kitten.

In the tetramethylene compounds (see Table I, where $n=4$), the presence of one ethyl group on each nitrogen caused a tenfold increase in activity on the superior

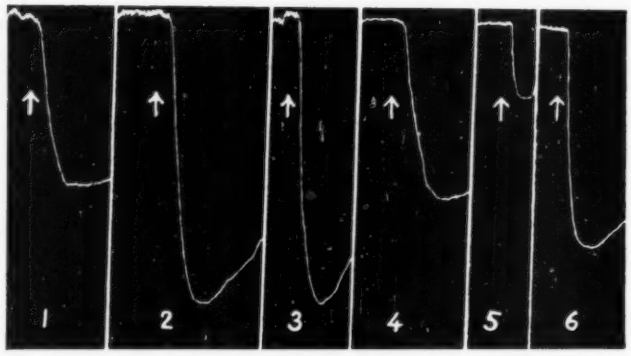


FIG. 1.—Cat, chloralose. Contractions of nictitating membrane on preganglionic stimulation of cervical sympathetic at 12 shocks/sec.; fall indicates relaxation. Compounds, as bromide salts, injected intravenously. (1) 0.25 mg., (3) 0.5 mg. hexamethonium; (2) 0.5 mg. tetramethylene-1:4-bis-diethylmethylammonium; (4) 10 mg. tetramethylene-1 : 4-bis-triethylammonium; (5) 50 mg. tetramethylene-1 : 4-bis-trimethylammonium; (6) 4 mg. tetramethylene-1 : 4-bis-ethyldimethylammonium. See text and Table I. Note that the effects in (2) and (3) are similar.

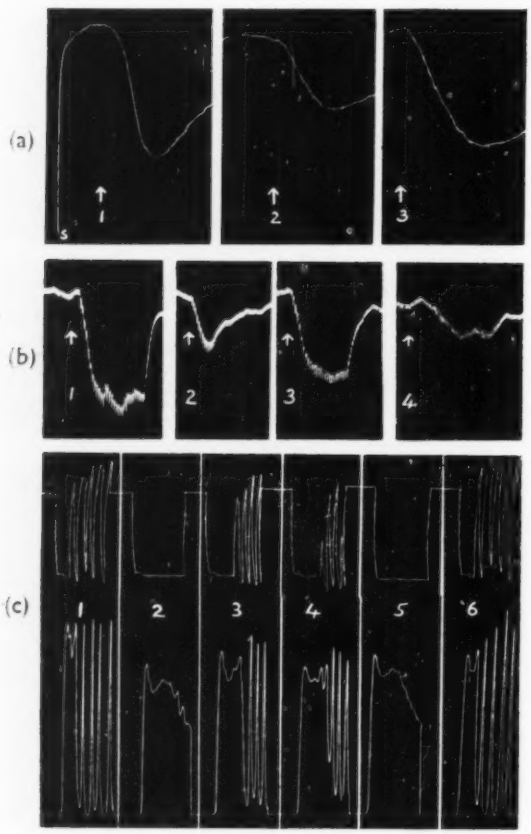


FIG. 2.—(*a*) Nictitating membrane contractions of cat (chloralose) on sustained preganglionic stimulation at S. (1) 0.2 mg., (2) 0.1 mg. intravenously of tetramethylene-1 : 4-bis-diethylmethylammonium, and (3) 0.2 mg. of hexamethonium bromides. (*b*) Blood pressure record of cat. (1) and (3) control effects on stimulation of peripheral end of severed right vagus; (2) and (4) after 0.5 mg./kg. intravenously of tetramethylene-1 : 4-bis-diethylmethylammonium and 0.25 mg./kg. of hexamethonium respectively. (*c*) Peristaltic responses of guinea-pig ileum; upper circular, and lower longitudinal, movements. (1) and (6) control effects; (2) 0.5 mg. and (3) 0.2 mg. of pentamethonium; (4) 0.5 mg. and (5) 1.0 mg. of tetramethylene-1 : 4-bis-diethylmethylammonium bromides.

cervical ganglion. The symmetrical replacement of two more methyl groups by ethyl caused a further tenfold increase, making it 100 times as active as the parent compound. This compound (tetramethylene-1 : 4-bis-diethylmethylammonium dibromide) was consequently as active as hexamethonium (Fig. 1). This compound, too, showed some selectivity in its action, since it had a relatively greater effect on the superior cervical ganglion than on the intestine and the vagus (Fig. 2). From Fig. 2 it will be seen that it was (*a*) as active as C6 on the superior cervical ganglion—the onset was quicker, but the duration was similar ; (*b*) about one-half as effective in preventing the fall of blood pressure produced by stimulation of the cardiac vagus ; and (*c*) about one-half as effective as C5 (equal to 37.5 per cent of C6) on the guinea-pig ileum.

There was a marked decrease in ganglion blocking potency in all the bis-triethylammonium compounds, but there was an increase in potency in the bis-ethyldimethylammonium homologues of penta- and hexa-methonium, in which only one methyl group on each nitrogen atom was replaced by ethyl (Wien and Mason, 1951).

Homologues of tetraethylammonium

Though the neuromuscular paralysing actions of several ethyl homologues of tetramethylammonium (Me_4N^+) have been studied by Marshall (1914), Burn and Dale (1914), Ing (1936), and Raventós (1937), and Dallemagne and Philippot (1951) have investigated a series Me_3RN^+, where $R = C_n H_{2n+1}$ and $n = 2$ to 18, we are unaware of any comparative data on the ganglion paralysing activities of these compounds, studied in the manner described in this paper. We have examined the effects on autonomic ganglia, and an illustration of the results obtained is shown in Fig. 3. In these experiments we found that tetraethylammonium (Et_4N^+) was

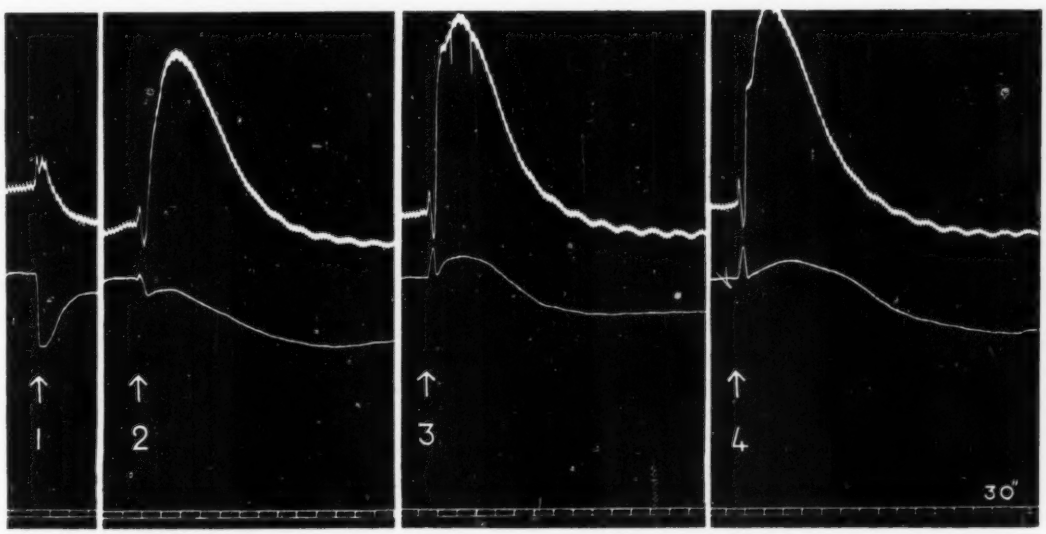


FIG. 3.—Cat, chloralose. Upper record, blood pressure. Lower record, contractions of nictitating membrane on sustained preganglionic stimulation; upstroke is contraction, downstroke is relaxation. Intravenously, (1) 5 mg. tetraethylammonium bromide; (2) 20 mg. diethyldimethylammonium bromide; (3) 5 mg. ethyltrimethylammonium bromide; (4) 2 mg. tetramethylammonium bromide.

between five and seven times less potent than hexamethonium. Ethyltrimethylammonium ($EtMe_3N^+$) had a marked nicotine-like pressor effect (comparable with tetramethylammonium) in doses which partially blocked ganglionic transmission to the nictitating membrane ; its effect was different from that of tetraethylammonium, and similar to that of tetramethylammonium. A stimulant action was observed also on the longitudinal muscle of the guinea-pig ileum, at all concentrations which caused an inhibition of the peristaltic movements. Triethylmethylammonium (Et_3MeN^+) had about the same ganglion blocking activity (one-quarter of Et_4N^+) as diethyldimethylammonium ($Et_2Me_2N^+$), but it was devoid of the stimulant actions of the latter compound, and its depressor effect was even more transient than that of tetraethylammonium.

γ-Phenoxypropyltriethylammonium and its trimethyl homologue

The triethylammonium compound was found by Depierre and Jacob (1950) to be about three times as potent as tetraethylammonium in paralysing the effects of nicotine on the blood pressure in dogs. We have found in the cat (chloralose) that it was one-half as potent as hexamethonium in paralysing transmission through the superior cervical ganglion ; its action, however, was much more evanescent. On the guinea-pig ileum preparation it was equally as active as hexamethonium in reducing the peristaltic reflex. This compound, like hexamethonium, affected only pre-ganglionic excitation, leaving post-ganglionic stimulation unmodified. Its trimethyl homologue, γ-phenoxypropyltrimethylammonium bromide, was almost devoid of any ganglionic paralysing effect (Fig. 4) ; consequently in these two mono-quaternary salts the complete replacement of methyl by ethyl groups increased ganglionic paralysing activity, whereas the reverse was true in the bis-quaternary ammonium compounds examined.

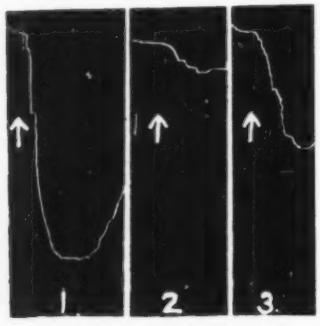


FIG. 4.—Cat, chloralose. Contractions of nictitating membrane on preganglionic stimulation. (1) 0.2 mg., (3) 0.1 mg. γ-phenoxypropyltriethylammonium iodide; (2) 1.0 mg. γ-phenoxypropyltrimethylammonium bromide.

DISCUSSION

We have found that in the alkamethonium series the type of terminal groupings on the two quaternary nitrogen atoms, as well as the intervening chain length, modifies ganglion blocking activity. This is illustrated in Fig. 5. Whereas increase in chain length from four to six carbon atoms caused a marked increase in potency, the same increase could be achieved by partial replacement of methyl by ethyl groups on the quaternary nitrogen atoms. It may well be that the most important factor is the preservation of the integrity of the terminal, basic polar groups ; any change in the quaternary nitrogens, for example, to tertiary nitrogens, results in an almost complete (95 per cent) reduction in activity. The characteristic feature, then, would

appear to be two cationic groups connected by an inert polymethylene chain. The association of biological activity with a quaternary nitrogen atom has been frequently observed; besides the paralysis of ganglia, such properties as neuromuscular paralysis and even activity against certain bacteria and trypanosomes have been observed. This might be due to some special property of the quaternary ammonium ion (for example, Lorente de Nó (1949) has shown its ability to replace sodium ions for the maintenance of the excitability of frog nerve), and indeed it is known that an enormous increase in basicity occurs on passing from a tertiary to a quaternary ammonium hydroxide, so that the positive charge associated with the quaternary ion might be responsible for an electrostatic attraction between it and the anion of the receptor grouping, particularly if the latter is regarded as of a weakly acidic nature.

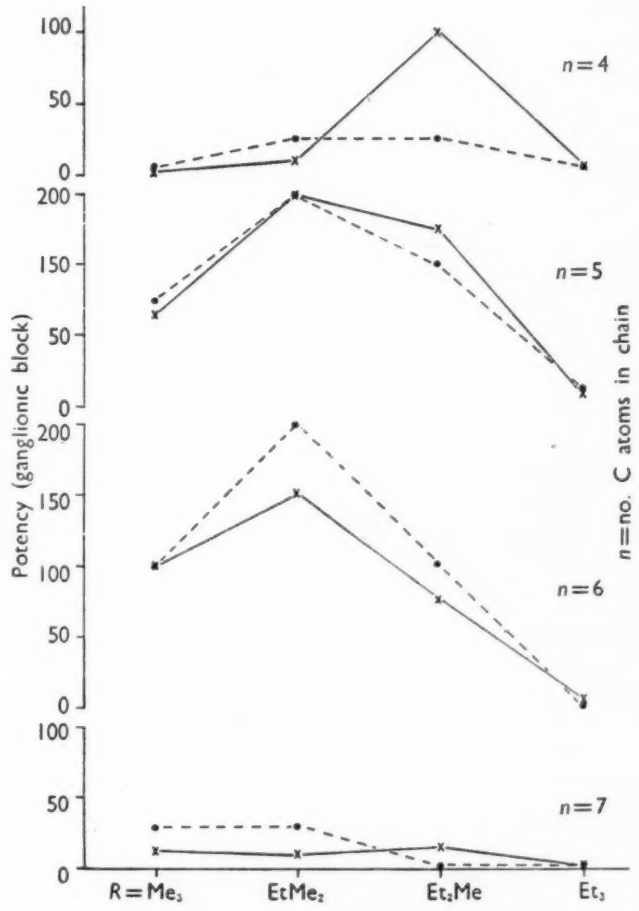


FIG. 5.—Relationship between ganglion blocking potency, chain length (n), and terminal groups (R) in the series $R.N^+(CH_2)_nN^+.R$; the maximal potencies vary with both n and R. × ——— × relative potencies on cat superior cervical ganglion. • - - - - • relative potencies on guinea-pig ileum.

We have not attempted to determine the exact mode of action of each compound, and it is conceivable that some of these compounds may indeed have mixed actions. Nevertheless, the substitution of one ethyl group for a methyl group on both nitrogen atoms was most effective where the intervening chain length was five or six carbon atoms, whereas the substitution of two ethyl groups was most effective where the chain

length was four carbon atoms. Tetramethylene-1 : 4-bis-diethylmethylammonium had a greater effect on the superior cervical ganglion relative to its effect on the intestine, showing that a ganglion blocking compound of high potency can be obtained with an affinity for a particular ganglionic system. But one of us (N.D.E.), in a quantitative study of drugs producing mydriasis in the mouse by paralysis of the ciliary ganglion, found this compound to be not less but 2.9 times more potent than hexamethonium, which may indicate a true differential action within the parasympathetic system or merely a difference due to species variation. With a chain length of seven carbon atoms substitution on the terminal nitrogens made little difference except to increase the neuromuscular blocking properties.

Two homologues of tetraethylammonium, namely diethyldimethyl- and ethyltrimethyl-ammonium bromides, displayed marked nicotine-like properties ; these compounds exemplify the well-recognized transition in properties between tetraethyl- and tetramethyl-ammonium, originally described by Burn and Dale (1914). The active ganglion blocking compound, γ-phenoxypropyltriethylammonium iodide, and its corresponding inactive trimethyl homologue, may be compared with the difference between tetraethylammonium and tetramethylammonium, all mono-quaternary compounds. In the bis-quaternary series the reverse was true, the complete substitution of methyl by ethyl groups rendering the compounds less effective. One explanation for this might be the existence of different receptors for the mono- and bis-quaternary ammonium compounds. Though ganglion blocking activity in the bis-quaternary compounds was very sensitive not only to the length of the alkyl chain, but also to the types of groups on the quaternary nitrogen atoms, it does not necessarily follow—since there is no direct evidence—that there is a corresponding optimal distance between the receptors. Moreover, the molecules are flexible and may assume various configurations within limitations. We would suggest, nevertheless, that an analysis of the electron configuration in the compounds we have examined might help to explain our findings ; obviously more information is wanted. An attractive hypothesis put forward by Taylor (1951) for the difference in curariform activity of Me_4N^+ and Et_4N^+ rests on differences in electron densities, and we think that similar deductions in the alkamethonium series might reveal values associated with changes in ganglion blocking activity.

Summary

1. The ganglion blocking properties have been studied of (*a*) a series of sixteen homologous compounds in the alkamethonium series in which methyl groups were successively replaced by ethyl groups on the two quaternary nitrogen atoms ; (*b*) three homologues of tetraethylammonium ; (*c*) γ-phenoxypropyltriethylammonium and its trimethyl homologue.

2. The potency of all these compounds was considerably influenced by the relatively minor change of ethyl for methyl groups on the quaternary nitrogen atoms.

3. In the alkamethonium series complete replacement of all the methyl by ethyl groups invariably led to a reduction in activity. Partial replacement caused an increase in potency, maximum at a chain length of four carbon atoms with the replacement by two ethyl groups on each nitrogen atom ; and at a chain length of five or six carbon atoms with the replacement by one ethyl group on each nitrogen

atom. Ganglion blocking activity was related not only to chain length but also to the type of terminal grouping. This was remarkably illustrated in the tetramethylene compounds, where the substitution of two ethyl groups produced as sharp and as great an increase in potency as alteration in chain length. The bearing of this on structure-action relationships in this series has been discussed, the observations not readily falling into line with any current theories. The alteration in potency resulting from alteration in structure usually occurred *pari passu* on both sympathetic and parasympathetic ganglia.

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THE FALL OF BLOOD PRESSURE CAUSED BY INTRAVENOUS MORPHINE IN THE RAT AND THE CAT

BY

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In 1933 Schmidt and Livingstone attempted to determine the cause of the marked fall of blood pressure which results when morphine sulphate is injected intravenously into the cat or the dog. They showed that the phenomenon exhibited tachyphylaxis and that it was unaffected by ether, urethane, barbitone, or phenobarbitone. They were unable to arrive at any definite conclusions and could only suggest that " depression of the vasomotor centre plays some part in this effect, but most of it is due to dilatation of cutaneous and muscular blood vessels by direct action upon their walls."

Sollmann and Pilcher (1917) reported that morphine evoked the triple response in the human skin, an observation which was confirmed by Lewis (1927). Since that time Nasmyth and Stewart (1950) have shown that the weals caused by morphine in human skin are reduced by antihistamine drugs. Feldberg and Paton (1950) reported that the intra-arterial injection of morphine into the isolated perfused gastrocnemius muscle of the cat caused the appearance of histamine in the effluent, and later (Feldberg and Paton, 1951) showed that the drug also released histamine from the cat's skin and that after its intravenous injection into the intact animal the plasma histamine was raised. They considered that the fall of blood pressure caused by the intravenous injection of morphine could not be wholly accounted for by the release of histamine.

In this work we have shown that the effect is complex and that at least three factors which would affect the blood pressure are involved when morphine is injected intravenously.

METHODS

Anaesthesia.—All the rats from which records of blood pressure were taken were anaesthetized with urethane (7 ml./kg. of a 25 per cent (w/v) solution injected subcutaneously). Rats which received injections of morphine sulphate into the jugular vein 24 hours before records of blood pressure were taken from them were anaesthetized with ether while the vein was exposed.

Cats were anaesthetized with ether followed by 80 mg./kg. of chloralose intravenously.

Records of blood pressure.—All animals except decerebrate and spinal cats, from which records of blood pressure were taken, were heparinized. The apparatus described by Condon (1951) was used to record the blood pressure of the rats.

Collection of adrenal vein blood samples.—The samples of cats' adrenal vein blood were collected by the method described by Vogt (1943), except that the colon was not removed. The samples were placed on ice as soon as collection was completed.

Assay of pressor substances in adrenal vein blood samples.—The effects of injecting suitable volumes of the adrenal vein blood samples were compared with those produced by doses of adrenaline on the cat's blood pressure. The animals were pretreated with 0.5 mg./kg. of atropine sulphate intravenously, 5 mg./kg. of mepyramine maleate subcutaneously, and 3 mg./kg. of cocaine hydrochloride intravenously. Thirty minutes later 40 mg. hexamethonium bromide was given intravenously. The blood samples were assayed immediately after the dose of hexamethonium bromide.

Number of experiments.—Each experiment was repeated with similar results in not less than three animals unless otherwise stated.

Drugs.—Throughout this paper the doses for drugs refer to the following salts: morphine sulphate, histamine acid phosphate, hexamethonium bromide, mepyramine maleate, atropine sulphate, acetylcholine chloride, and nicotine hydrogen tartrate.

RESULTS

Intravenous injection of morphine in rats

Anaesthetized rats.—In the initial experiments 10 mg./kg. of morphine was injected into the jugular vein. This dose always produced a sharp transient fall in blood pressure lasting from 20 to 30 seconds. Upon repeating the injection the result varied from a less marked but equally transient fall to no effect; this tachyphylaxis occurred consistently. A third dose given shortly after the second was invariably without effect. It was subsequently found that 10 mg./kg. was an unnecessarily high dose and that similar responses could be obtained with doses of 1 mg./kg.

The fall of blood pressure caused by morphine was more transient than that caused by an equi-depressor dose of histamine. Whereas the response to morphine exhibited tachyphylaxis, the response to repeated injections of the same dose of histamine remained unchanged. This is illustrated in Fig. 1.

An attempt was made to determine the period during which the vasodepressor response to intravenous morphine was unobtainable after initial doses of the drug. It was found impossible to keep the rats in a suitable condition for experiment for longer than five hours when their blood pressure was being recorded from the

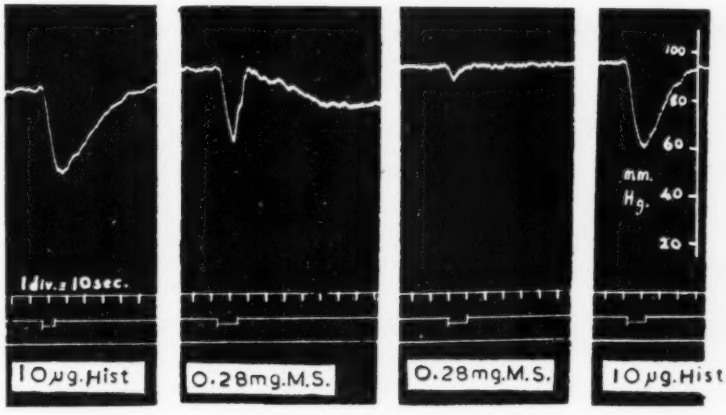


FIG. 1.—Rat: Urethane. The effect of repeated intravenous injections of 1 mg./kg. of morphine on blood pressure. The tracings run consecutively; parts of the record have been removed to condense the material. They show the transient response to morphine compared with the responses to histamine, when both are injected intravenously. Note the tachyphylaxis displayed by the response to morphine. Hist. = Histamine. M.S. = Morphine sulphate.

carotid artery, but in experiments covering this period, a further fall in blood pressure was only obtained once, and then only two hours after the first ineffective dose of morphine. It was concluded that the vasodepressor response is not generally repeatable until more than five hours have elapsed after the last effective dose of morphine.

A number of experiments were performed in which three 1 mg./kg. doses were injected into the exposed jugular vein with a three-minute interval between each dose. The wound was then sutured and the animal used 24 hours later to record blood pressure in the usual way. In all these animals a vasodepressor response was obtained when 1 mg./kg. of morphine was injected intravenously. It was concluded that the normal response to intravenous morphine in the rat, after the vasodepressor response has once been elicited, cannot be obtained until more than 5 but less than 24 hours have elapsed. It was noted that the second series of morphine injections causing falls in blood pressure also exhibited tachyphylaxis.

Rats treated with mepyramine.—Nasmyth (1951) showed that the subcutaneous injection of 5 mg./kg. of mepyramine into rats effectively blocked the fall of blood pressure caused by intravenously injected histamine for periods in excess of 3 hours. The same dose and route of injection were therefore employed in these experiments. The rat is relatively insensitive to histamine and intravenous doses of 10 μg. histamine were required to produce appreciable responses. Twenty to thirty minutes after the injection of mepyramine, no response could be obtained to the intravenous injection of 30 μg. histamine. The intravenous injection of 1 mg./kg. of morphine under these circumstances caused a fall of blood pressure which was more profound and less transient than that obtained in animals untreated with mepyramine. Typical records are shown in Fig. 2.

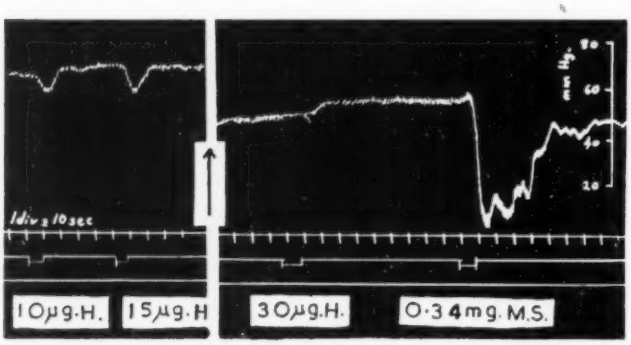


FIG. 2.—Rat: Urethane. The effect of mepyramine on the response of the blood pressure to 1 mg./kg. of morphine sulphate injected intravenously. At the arrow 5 mg./kg. of mepyramine was injected subcutaneously and the morphine was injected 30 minutes later. H = Histamine. M.S. = Morphine.

Vagotomized or atropinized rats.—The left and right vagi were sectioned in a series of rats before 1 mg./kg. of morphine was injected into the jugular vein. On each occasion a small rise of pressure occurred in place of the usual fall. This is illustrated in Fig. 3A.

In view of this finding, the effect of atropine was investigated. Accordingly 0.25 mg. atropine was injected intravenously, sufficient to abolish the blood pressure response to 5 mg. of intravenous acetylcholine. The subsequent injection of 1 mg./kg. of morphine intravenously still caused a fall of blood pressure, but the blood pressure did not return to its original level. Subsequent doses of 1 mg./kg. of morphine exhibited the usual tachyphylaxis in the hypotensive response. These effects are

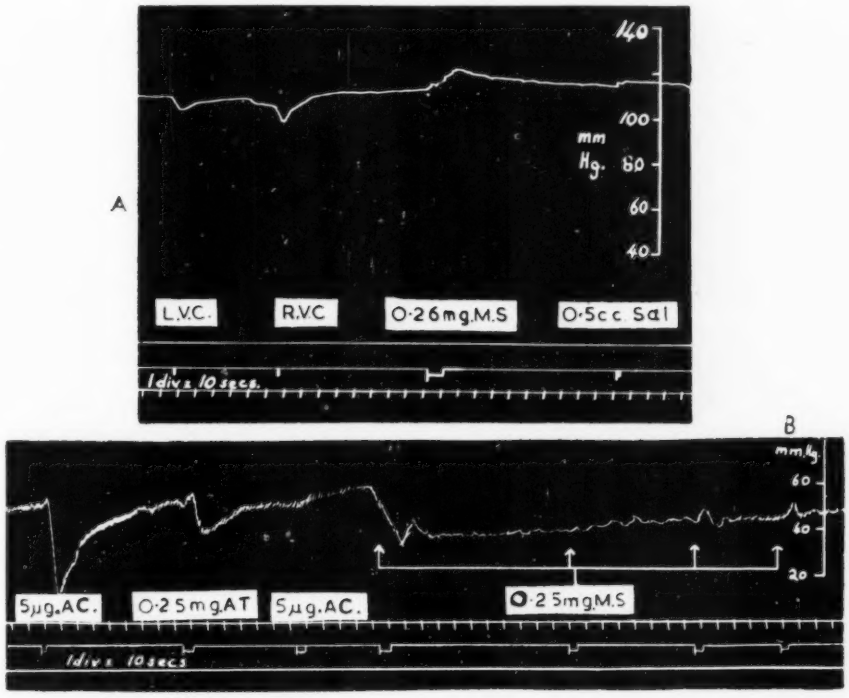


FIG. 3.—Rat: Urethane. The effect of the intravenous injection of 1 mg./kg. of morphine on blood pressure:—Tracing A—after cutting both vagi. Tracing B—after atropine. R.V.C.=right vagus cut. L.V.C.=left vagus cut. M.S.=Morphine. AC.=Acetylcholine. AT.=Atropine.

illustrated in Fig. 3B. A dose of 2 mg. acetylcholine, after these doses of morphine, caused a rise in blood pressure which indicated that morphine had no effect on the action of acetylcholine on sympathetic ganglia.

Rats treated with hexamethonium.—The dose of hexamethonium required to produce ganglion block in rats was unknown. When the elevation of blood pressure produced by the intravenous injection of 0.1 mg. nicotine was used as a criterion, it was found that 60 mg./kg. of hexamethonium was required to produce ganglion block. Under these circumstances responses to intravenous injections of morphine were blocked at dose levels of 1 mg./kg. and at 10 mg./kg. The result is illustrated in Fig. 4.

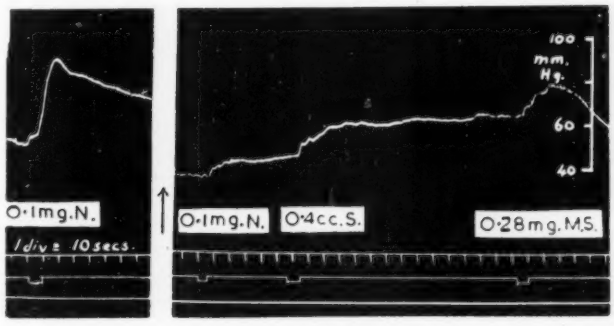


FIG. 4.—Rat: Urethane. The effect of the intravenous injection of 1 mg./kg. of morphine on blood pressure after 60 mg./kg. of hexamethonium given intravenously (at the arrow). N=Nicotine. S=Normal saline. M.S.=Morphine.

The intravenous injection of morphine in cats

Anaesthetized cats.—The effect of intravenously injected morphine on the cat's blood pressure differs in some respects from the effect obtained in the rat. Pilot experiments indicated that a fall of pressure was sometimes obtained when 2 mg./kg. of morphine was injected into the jugular vein, but that 4 mg./kg. was always effective.

The intravenous injection of the latter dose caused a transient rise in pressure followed by a precipitous fall 15 to 20 seconds after completion of the injection. During the first 2 or 3 minutes after the injection, the heart rate increased slightly but was subsequently slower than normal. After the pressure had fallen, it did not recover during the next seven hours. The infusion of 50 ml. of 3.5 per cent polyvinylpyrrolidone at 37° C. at the end of this period served to raise the pressure only a little. A second dose of 4 mg./kg. of morphine then caused a transient fall in pressure. A third dose gave a yet smaller response. At this dose level the respiration was always inhibited, and sometimes failed, but whether artificial respiration was employed or not the fall in blood pressure was constant.

When doses of 20 mg./kg. were injected intravenously in anaesthetized cats, the pattern of the blood pressure response was identical with that obtained with a dose of 4 mg./kg. With the larger dose the fall in pressure was more profound, often to 20 or 30 mm. of mercury, but within 2 hours it had risen and remained at a level corresponding with that to which the pressure usually fell with the smaller dose. Repetition of the dose of morphine under these circumstances produced transient and gradually diminishing responses. The respiration always failed when 20 mg./kg. of morphine was injected intravenously.

Decerebrate cats.—In the decerebrate cat, the intravenous injection of 4 mg./kg. of morphine caused a fall of blood pressure which was less precipitous but just as profound as that produced by the same dose of the drug in the anaesthetized cat. The effect was of shorter duration, lasting 10 to 15 min., and exhibited the usual tachyphylaxis on repeated injections. In these animals the respiration never failed and was rarely more than very slightly depressed.

Spinal cats.—When 4 mg./kg. of morphine was injected intravenously in the spinal cat, the fall in blood pressure was precipitous, but less profound than in the anaesthetized cat, presumably because the vasomotor tone was lower. Within 40 sec. of completion of the injection the pressure began to rise, and after 70 sec. it had returned almost to the initial level. The initial pressure level was usually regained within 15 min., and a second dose of 4 mg./kg. of morphine then gave a similar but smaller effect and repeated dosage with morphine eventually abolished it. In one animal the fall in blood pressure disappeared after five injections of morphine given during a period of 80 min., but when a sixth injection of morphine was given, 30 min. after the fifth, a typical fall of pressure was obtained. In another spinal animal the initial pressure was only 45 mm. of mercury, and in this instance morphine produced no blood pressure response. The effects of the intravenous injection of 4 mg./kg. of morphine in anaesthetized, decerebrate, and spinal cats are illustrated in Fig. 5.

Cats treated with mepyramine.—A dose of 5 mg./kg. of mepyramine was injected subcutaneously into anaesthetized cats. Usually it blocked the depressor response to doses of histamine ranging from 1 to 5 μg. 30 min. after the administration. A few

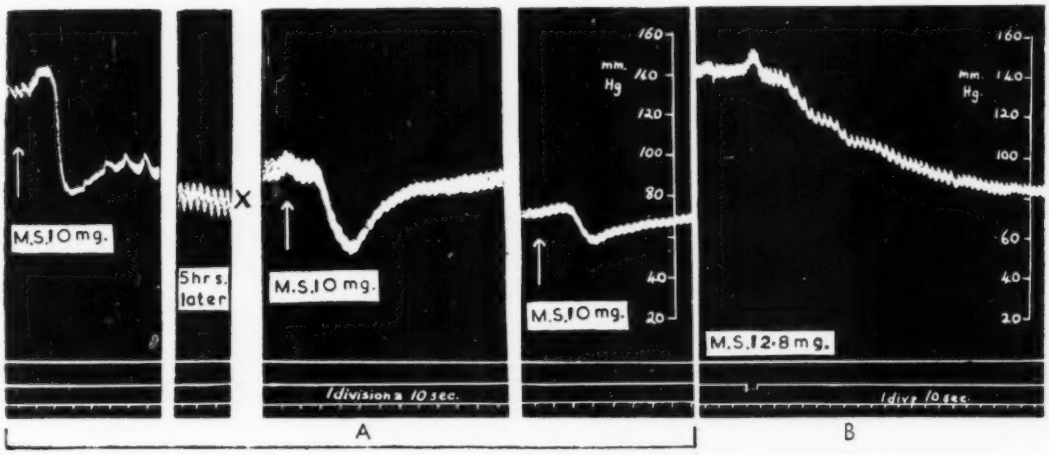
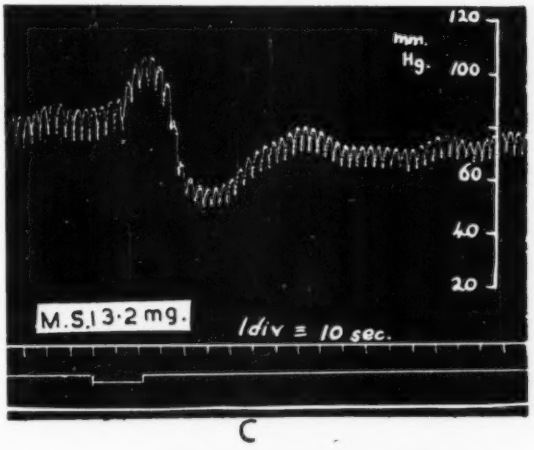


FIG. 5.—The effects on blood pressure of 4 mg./kg. of morphine injected intravenously in the cat. (A) Anaesthetized cat (chloralose): 50 c.c. of polyvinylpyrrolidone (3.5 per cent) was infused at X. There was an interval of 5 min. between the last two doses of morphine. (B) Decerebrate cat. (C) Spinal cat. M.S.= Morphine.



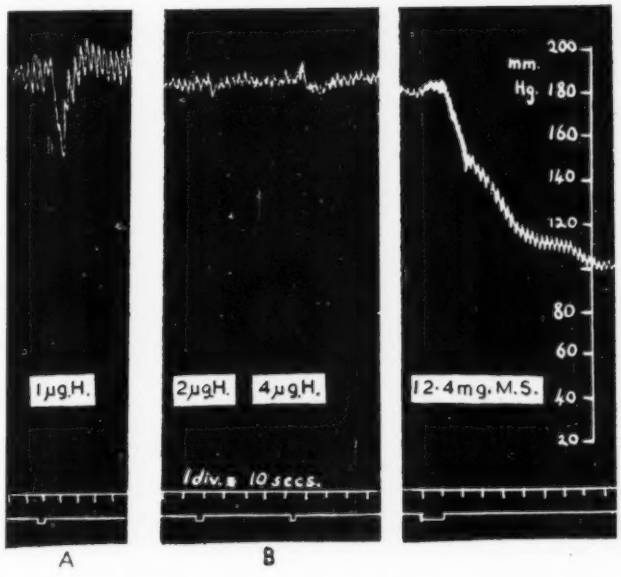


FIG. 6.—Cat: Chloralose. The effect of mepyramine on the fall of blood pressure caused by injecting 4 mg./kg. of morphine intravenously. There was an interval of 30 minutes between (A) and (B), at the commencement of which 5 mg./kg. of mepyramine was injected subcutaneously. H = Histamine. M.S.= Morphine.

animals were resistant to mepyramine and even intravenous injection of the drug failed to block the blood pressure response to histamine. These animals were discarded.

After an effective dose of mepyramine maleate, 4 mg./kg. of morphine sulphate injected intravenously always produced a profound and prolonged fall of blood pressure.

In two decerebrate cats similarly pretreated with mepyramine the pressure fell when 4 mg./kg. of morphine was injected intravenously, but the magnitude of the response was less than half that normally obtained in decerebrate cats. In a third animal pretreated with mepyramine, the intravenous injection of 4 mg./kg. of morphine sulphate produced no response. The effect in the spinal cat was always completely blocked by pretreatment with mepyramine.

The effect of intravenously injected morphine in the anaesthetized cat pretreated with mepyramine is illustrated in Fig. 6.

Atropinized and vagotomized cats.—In order to determine the effect of atropine on the phenomenon the left vagus was cut and the effect of stimulating the peripheral cut end was investigated ; 5 mg. atropine was then injected, and, after a few minutes, stimulation of the peripheral cut end of the left vagus was without effect. The injection of 4 mg./kg. of morphine then caused the usual fall of blood pressure and it remained low for two hours. In cats with both vagi cut, the pattern of the response to an intravenous injection of 4 mg./kg. of morphine was not modified.

Cats treated with hexamethonium.—The intravenous injection of 0.1 mg. nicotine produced a steep rise in blood pressure. After a dose of 40 mg. hexamethonium intravenously, injection of 0.3 mg. nicotine gave no pressor response ; this was taken as evidence that the autonomic ganglia were effectively blocked. A dose of

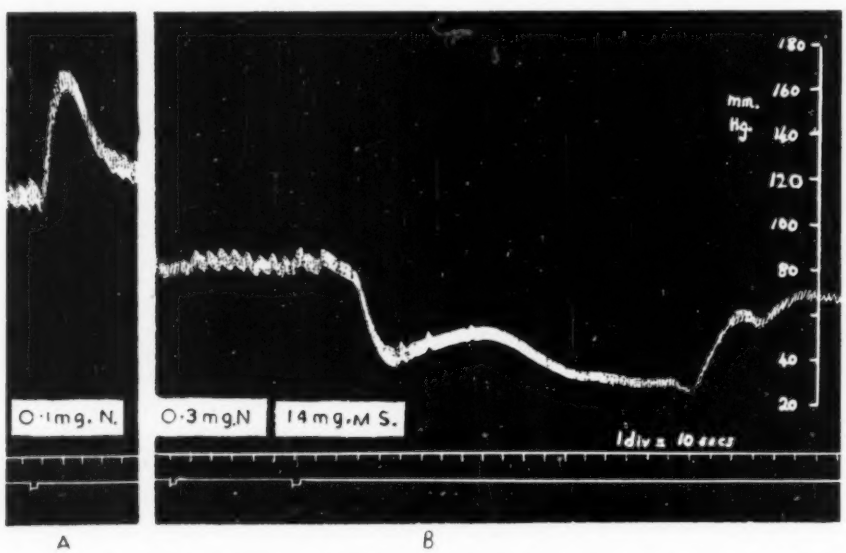


FIG. 7.—Cat: Chloralose. The effect of hexamethonium on the fall of blood pressure caused by injecting 4 mg./kg. of morphine intravenously. During the interval between (A) and (B) 10 mg./kg. of hexamethonium was injected intravenously. N=Nicotine. M.S.=Morphine.

4 mg./kg. of morphine now caused a precipitous fall in blood pressure, which in two experiments was more transient than that normally obtained. In one animal the pressure had returned to a level only 10 mm. of mercury below the initial value within four minutes (Fig. 7). The effect in the second animal was similar, but it was twelve minutes before the pressure approximated to the normal value. In the third animal the injection of morphine caused the usual fall in blood pressure, which remained low. In this series of experiments 4 mg./kg. of morphine almost invariably stopped the respiration.

It seemed possible that the more transient fall in blood pressure obtained in the presence of hexamethonium could be due to histamine released from tissues. In order to test this point, a series of experiments was performed in which the animals were pretreated with mepyramine and hexamethonium. The responses to 0.1 mg. nicotine and 2 μg. histamine were first demonstrated; thereupon 5 mg./kg. of mepyramine was injected subcutaneously, followed by 40 mg. hexamethonium intravenously thirty minutes later. Repetition of the doses of nicotine and histamine then produced no change in the blood pressure. Following this, 4 mg./kg. of morphine injected intravenously caused a very slow fall in blood pressure as shown in Fig. 8.

Pressor substances in the adrenal vein blood after intravenous morphine

The preliminary rise of blood pressure which was always observed when morphine was injected into anaesthetized, but otherwise untreated, cats suggested that there might be a considerable release of pressor substances from the adrenal medulla. Consequently, an experiment was performed in which the total pressor activity of

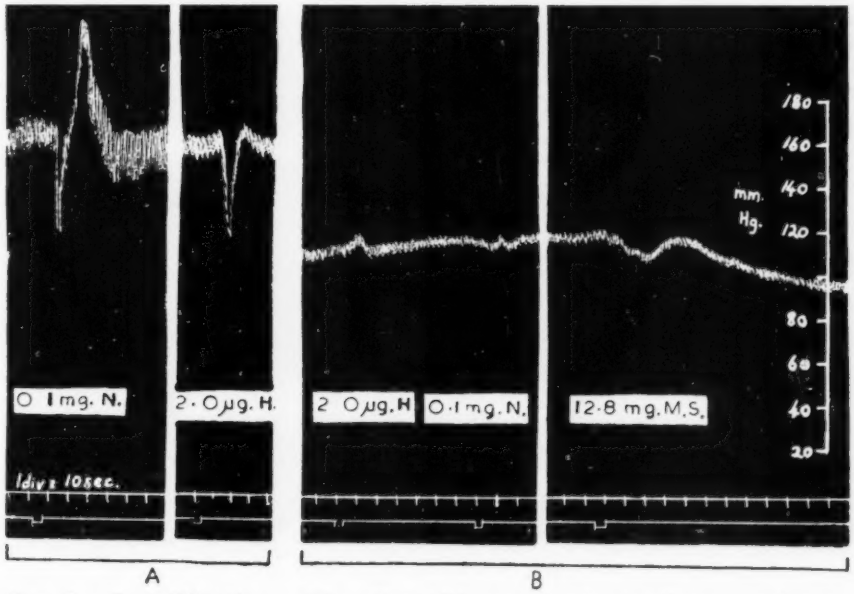


FIG. 8.—Cat: Chloralose. The effect of hexamethonium and mepyramine on the fall of blood pressure caused by injecting 4 mg./kg. of morphine intravenously. There was an interval of 30 minutes between A and B, at the commencement of which 5 mg./kg. of mepyramine was injected subcutaneously. At the end of this period, 10 mg./kg. of hexamethonium was injected intravenously. N = Nicotine. H = Histamine. M.S. = Morphine.

TABLE I

The pressor activity (in terms of μg. of *l*-adrenaline per ml.) of cat's adrenal vein blood samples before and after the intravenous injection of morphine. Collection of the first sample after morphine began immediately, and there were no intervals between the collection periods of the following samples.

Cat	Sample volume (ml.)	Dose of morphine (mg./kg.)	Collection period (min. sec.)	Pressor activity (μg. adrenaline per ml.)
A	5.0	—	2 20	0.25
	2.5	4	5 10	1.74
	5.0	4	3 45	3.73
	5.0	4	3 20	2.40
B	5.0	—	1 5	0.31
	5.0	4	2 28	3.80
	5.0	4	4 15	2.00
	5.0	4	3 20	1.70
C	5.0	—	1 35	0.20
	5.0	4	3 35	2.00
	5.0	4	3 0	2.00
D (Control)	5.0	—	1 20	0.40
	5.0	—	1 5	0.40
	5.0	—	1 10	0.60
	5.0	—	1 23	0.45

adrenal vein blood samples was assessed before and after an intravenous injection of 4 mg./kg. of morphine.

The samples of adrenal vein blood were collected and assayed as already described. With one exception the volume of each sample was 5 ml. The results obtained are shown in Table I. In each of the cats A, B, and C, one sample was collected after a control injection of normal saline; 4 mg./kg. of morphine was then injected into the jugular vein and the collection of further samples started immediately. It will be noted that in these cats the collection periods and the pressor activity of the samples were increased after the injection of morphine. In cat A, the collection period of the first sample after the injection of morphine was so much increased that only 2.5 ml. had been obtained in 5 min. and 10 sec.

In order to determine the effect of withdrawing 20 ml. of blood on the pressor activity of the adrenal vein blood, an experiment was performed in which four 5 ml. samples were collected without the injection of morphine. The results obtained in this experiment are shown for cat D. It will be observed that neither the pressor activity nor the collection period of the samples was significantly altered.

Discussion

The observations of Schmidt and Livingstone (1933) on the depressor effect of intravenous morphine on the cat's blood pressure have been confirmed and extended.

In the spinal cat the intravenous injection of 4 mg./kg. of morphine caused a small transient rise in blood pressure followed by a precipitous fall from which recovery was complete within fifteen minutes, and the phenomenon was shown to exhibit tachyphylaxis on repetition of the dose. The effect could not be obtained in animals with a pressure lower than 60 mm. Hg. In cats anaesthetized with chloralose the

pattern of the response was the same as that in the spinal cat, except that the fall in pressure was more profound and persisted for longer than five hours. This difference suggested that either the anaesthetic or the medullary and hypothalamic centres contributed to the effect in the anaesthetized animal. In the decerebrate cat the fall of blood pressure caused by morphine was less precipitous than, but equally profound as, that in the anaesthetized animal. It was, however, of shorter duration, lasting only 10–15 minutes. The similarity in the duration of the effect in spinal and decerebrate cats and the prolongation of the phenomenon in animals under chloralose suggested that the anaesthetic either potentiated the blood pressure lowering effect of the morphine or inhibited compensatory mechanisms.

Though morphine is known to release histamine from cat's skin and muscle, pretreatment of anaesthetized animals with mepyramine did not alter the response of the blood pressure to intravenously injected morphine, whereas similar pretreatment of spinal and decerebrate cats either abolished or considerably reduced the response. The inference was that the phenomenon in the spinal and decerebrate cats was mediated by histamine released in the animal's tissues, and that any central actions of morphine affecting blood pressure were compensated. The ineffectiveness of mepyramine in the anaesthetized cat suggested that morphine had some central action which was potentiated either by the anaesthetic or by vasomotor effects that were uncompensated in its presence.

The observation in two experiments that pretreatment of anaesthetized cats with hexamethonium did not abolish the fall in blood pressure, but reduced its duration to a time comparable with that seen in spinal and decerebrate animals, may indicate that morphine has some effect on central vasomotor mechanisms. It is interesting to note that this period corresponds closely with the time during which plasma histamine is increased after morphine injection (Feldberg and Paton, 1951). It was also shown that pretreatment of anaesthetized animals with both mepyramine and hexamethonium abolished the typical response of the blood pressure to intravenous morphine.

Some indication of the nature of the central effect was obtained from the observations that neither pretreatment with atropine nor bilateral vagotomy affected the pattern of the response to intravenous morphine in the anaesthetized cat. The additional observation, that the heart rate was increased during the first two or three minutes after a dose of morphine, also contributes to the supposition that the central actions of morphine which affect the circulation are on the vasomotor rather than the vagomotor centre.

Schmidt and Livingstone (1933) reported that morphine did not lower the blood pressure of the rat under ether, yet they show a kymograph tracing depicting an exactly similar fall of pressure to that reported by us. In addition to being different in character from the response in the cat, the response in the rat is also different in nature, since it is blocked by hexamethonium bromide and by bilateral vagotomy, but not by atropine. The inference would appear to be that the effect of intravenous morphine on the circulation of the rat is mediated by sensory impulses in the vagus nerve.

It was shown by Elliott (1912), and confirmed by Emmelin and Strömblad (1951), that morphine depletes the adrenal medulla of its content of pressor substances. The latter authors also showed that ether and chloralose anaesthesia had a rela-

tively small effect. The cats in which the pressor content of adrenal vein blood samples was assayed were pretreated with an antihistamine and atropine, as it had been shown by Schmiterlow (1951) that the presence of acetylcholine or histamine in samples to be assayed for pressor activity materially affects the result. Schmiterlow (1951) also recommended the removal of the adrenal glands from the test animal in order to prevent the release of pressor substances by contaminants which may be present in the samples. In the foregoing experiments the test animal was pretreated with hexamethonium which, at one and the same time, prevented the release of pressor substances and helped to make the animal more sensitive to those injected.

Summary

1. The intravenous injection of 4 mg./kg. of morphine causes a profound and prolonged fall of blood pressure in the cat anaesthetized with chloralose. The effect occurs, but is modified, in decerebrate and spinal cats. The effect in the rat anaesthetized with urethane is transient, it exhibits tachyphylaxis, and can be obtained with doses as low as 1 mg./kg. Once the effect has been exhausted in the rat, it can be repeated after 24 hours have elapsed.

2. The effect is not blocked by mepyramine in either anaesthetized cat or rat. In the spinal cat the effect is completely blocked by mepyramine, and in the decerebrate cat it is very much reduced.

3. Hexamethonium bromide prevents the effect in the rat. It sometimes modifies, but never blocks, the effect in the anaesthetized cat. The combination of hexamethonium and mepyramine minimizes the response in the cat.

4. Atropine does not prevent the response in either the rat or the cat.

5. Cutting the vagi abolishes the effect in the rat but not in the cat.

6. The content of pressor substances in adrenal vein blood samples from the cat is increased 10 to 15 times after the intravenous injection of 4 mg./kg. of morphine and remains high for at least 12 minutes after the injection.

7. It is concluded that the activity of intravenously injected morphine on the circulation of the cat is mediated largely, if not entirely, by an effect on the vasomotor centre and by the release of histamine peripherally. There is some compensation by increased secretion from the adrenal medulla. In the rat the effect appears to be mediated wholly by sensory impulses in the vagus nerve.

Our thanks are due to Professor Buttle, of the School of Pharmacy, for the loan of the Condon apparatus for recording rat blood pressures, and to Messrs. May and Baker Ltd. for generous supplies of polyvinylpyrrolidone and " Anthisan," and to the Sir Halley Stewart Trust for their continued financial support.

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A QUANTITATIVE STUDY OF ANTAGONISTS OF ADRENALINE ON THE VESSELS OF THE RABBIT'S EAR

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A large number of substances can act as antagonists of adrenaline on the vessels of the rabbit's ear. Among them are some agents, such as the ergot alkaloids, yohimbine, F933, and dibenamine, which are generally considered as specific inhibitors of adrenaline, but in addition there are others usually classed as antihistamine substances or as local anaesthetics, etc. It is well known that Bovet discovered antihistamine substances in a search for anti-adrenaline compounds, and Burn and Dutta (1948), working on rabbit ear vessels, found that procaine, atropine, quinidine, pethidine, and other substances were able to abolish the vasoconstrictor effect of adrenaline and of histamine. The vasoconstrictor effect of adrenaline was even reversed when the ears were perfused with quinidine, diphenhydramine (Benadryl), or procaine.

These findings showed that adrenaline is antagonized, on the isolated rabbit's ears, by many substances other than those which could be expected to act as inhibitors from the results of simple blood pressure experiments. Thus the question arose whether it was possible to make a clear distinction on the vessels of the rabbit ear between substances commonly regarded as having an anti-adrenaline action and those better known as having other properties, such as antihistamine and local anaesthetic action.

A quantitative study has therefore been made of the abolition of the constrictor action of adrenaline by various substances when perfused in Locke's solution through the rabbit ear vessels, and also of the rate of return of the constrictor action after perfusing once more with Locke's solution alone. The rabbit ear vessels have been found to be well suited to making quantitative studies of this kind when the outflow recorder of Stephenson (1948) was used.

It is increasingly believed that inhibition or reversal of the constrictor effect of adrenaline is due to competition by the inhibiting or reversing agent for the receptors to which adrenaline attaches itself. Presumably an inhibiting substance may be said to be specific when it has an especially high affinity for the adrenaline receptors. It should then block these receptor groups in higher dilution than that in which it blocks other receptors. Perhaps also the combination of the specific inhibitor with the receptor must be relatively stable and not quickly reversible.

METHOD

The experimental procedure was the same as in the previous work of Burn and Dutta (1948) and Burn and Robinson (1951). A fine glass cannula was tied into the central artery at the base of the ears severed from the head. Then the ears were perfused with Locke's solution, saturated with oxygen + 5 per cent CO_2, at room temperature and at a constant pressure which produced an outflow of about 3 ml./min. Injections of test doses of adrenaline were made into a small rubber-capped chamber, through which the perfusion fluid passed just before entering the cannula. These test doses of adrenaline were given in a constant volume of 0.1 ml. Locke's solution and varied from 0.001 to 0.05 μg., depending on the sensitivity of the ear vessels. The dose of adrenaline most frequently used was 0.01 μg. The final dilutions of adrenaline in Locke's solution were prepared just before each injection. As in the previous experiments of Burn and his colleagues, Stephenson's outflow recorder was found to be most satisfactory in registering very small changes of the vascular tone.

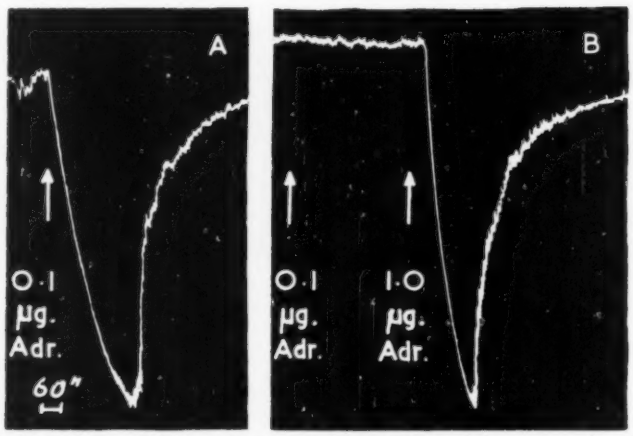

FIG. 1.—The anti-adrenaline activity of ergotoxine ethanesulphonate. A. Normal constrictor effect of 0.1 μg. adrenaline. B. A dose of adrenaline (1 μg.) 10 times greater than the initial test dose produces the same degree of constriction when ergotoxine ethanesulphonate (0.02 μg./ml.) has been perfused for 30 min.

When constant responses to several injections of a certain test dose of adrenaline had been obtained, perfusion with pure Locke's solution was discontinued and changed for Locke's solution containing an anti-adrenaline substance. Then the decrease of the vascular response to adrenaline was determined after 30 min. of perfusion. By a number of such experiments the exact concentration of substance was found which reduced the effect of adrenaline to 1/10 of the initial effect; thus after perfusion with an anti-adrenaline substance a dose of adrenaline 10 times greater than the initial test dose produced the same constriction as the initial test dose before perfusion, as shown in Fig. 1.

When pure Locke's solution was perfused again the initial sensitivity to adrenaline returned more or less quickly. The period required for complete recovery was determined in each experiment. The ears were generally used for two days, and several experiments were made in this time. As already observed by Burn and Dutta the vessels became more sensitive to adrenaline with continued perfusion so that at a later stage a given dose produced more constriction than before. The opposite change was never found. Thus a decrease of the constrictor effect of adrenaline was always due to the drug perfused. The potency of the anti-adrenaline substances, on the other hand, was about the same on the first and on the second day of perfusion and differed only slightly on different ears.

RESULTS

Comparison of the anti-adrenaline potency on the rabbit ear vessels.—The anti-adrenaline potency of various compounds was first investigated. Table I shows the results. Most of the substances commonly regarded as anti-adrenaline compounds were found to act in the lowest concentration. The most powerful were the substances " ergotoxine ethanesulphonate " (B.P. 1932) and ergotamine tartrate. Twelve experiments were performed. " Ergotoxine ethanesulphonate " (0.02 μg./ml.) and ergotamine tartrate (M/10,000,000) produced a considerable decrease of the outflow without completely blocking adrenaline. It was sometimes quite difficult to determine whether adrenaline was inhibited by these substances or whether its action on the ear vessels was no longer visible because they were so constricted. Nevertheless, in four experiments with 0.02 μg./ml. of " ergotoxine ethanesulphonate " in the perfusion fluid, a reduction of the adrenaline effect to about 1/10–1/5 was obtained, roughly comparable with the effect of the other adrenaline inhibitors; in these experiments the recovery took more than 100 min. These results are not included in Table I. After these ergot alkaloids, the substance Regitine (CIBA 7337; 2-(N′-*p*-tolyl-N′-*m*-hydroxyphenylaminomethyl)-imidazoline hydrochloride) was found to be the most potent, and next in order was dibenamine. The degree of inhibition with dibenamine was found to depend on the duration of the perfusion much more than with other substances. As a rule no further change in the action of adrenaline was obtained by prolonging the perfusion with the anti-adrenaline compound beyond 30 min. With dibenamine, however, the change was progressive,

TABLE I

ANTI-ADRENALINE EFFECTS OF VARIOUS DRUGS IN THE PERFUSED RABBIT-EAR PREPARATION

Substance	Molar concn. required to reduce constrictor action of adrenaline to 1/10th	No. of exps.	Time of washing required for complete recovery (min.)
Regitine (C7337)	M/6 million	7	60
Dibenamine	M/4 million	9	Almost irreversible in 20 hr.
Phenindamine (Thephorin)	M/640,000	4	25
Promethazine (Phenergan)	M/640,000	4	25
Yohimbine	M/320,000	10	120–180 (or more)
Lergigan (RP3389)*	M/320,000	4	15
F933	M/275,000	12	25–45
Antazoline (Antistin)	M/128,000	6	15
Tripelennamine (Pyribenzamine)	M/64,000	4	25
Mepyramine (Neoantergan)	M/32,000	12	20
Tolazoline (Priscol)	M/32,000	19	20
Quinidine	M/32,000	6	15–30
Quinine	M/32,000	4	30–45
Cinchocaine (Nupercaine)	M/32,000–M/16,000	6	20–30
Diphenhydramine (Benadryl)	M/16,000	9	40
Atropine	M/16,000	12	30–40
Pethidine	M/4,000	6	30–40
Amethocaine (Pontocaine)	M/2,000	4	20
Strychnine	M/2,000	11	25–30
Procaine	M/500	4	15
Cocaine	M/250	8	20

* Lergigan is regarded as being 10-(1′-dimethylamine-2′-propyl)-phenothiazine.

the anti-adrenaline action becoming more pronounced even after removing the dibenamine from the perfusing fluid; thus the concentration given in Table I for dibenamine must be regarded as approximate and was obtained when it had been applied for 30 min. The concentration would have been different if the time of perfusion had been different. The very high anti-adrenaline effect of the antihistamine compounds was most striking. Thus Phenindamine (Thephorin) and promethazine (Phenergan) were more active than yohimbine and F933. Other antihistamine compounds were weaker. It is interesting that a similar order for the anti-adrenaline activity of some of these antihistamine compounds was found on the isolated seminal vesicle of guinea-pigs (Haas, 1951). The comparison of the active concentration on the seminal vesicle with the present results on the ear vessels is shown in Table II.

TABLE II

ANTI-ADRENALINE POTENCY OF SOME ANTIHISTAMINE COMPOUNDS ON THE ISOLATED SEMINAL VESICLE OF GUINEA-PIGS AND ON THE RABBIT'S EAR VESSELS

Substance	Seminal vesicle conc. required for complete inhibition of adrenaline (Haas, 1951) (mg./litre)	Rabbit's ear vessels conc. required for reducing the constrictor effect to 1/10th (mg./litre)*
Promethazine	0.4	0.5
Antazoline	4.0	2.3
Tripelennamine	5.0	4.5
Mepyramine	10.0	12.0

* Absolute concentrations corresponding to the mol. concentrations of Table I.

2-Benzylimidazoline (tolazoline, Priscol), which is an anti-adrenaline substance, was about 20 times less active than Phenindamine or promethazine and was equal in potency to quinine, quinidine, and cinchocaine. Diphenhydramine and atropine were tested and found to be half as effective as tolazoline. From these observations it was clear that substances commonly regarded as specific inhibitors of adrenaline, such as F933 and tolazoline, do not always have a powerful action on the perfused rabbit's ear vessels.

Other drugs tested were pethidine, amethocaine (pontocaine), and strychnine, which proved to be antagonists of moderate potency. Weakest of all was cocaine; the inhibition produced by this substance was 2,000–3,000 times less than the inhibition exerted by phenindamine or promethazine. Procaine was twice as strong as cocaine. It is known that some antihistamine compounds may cause a short and sometimes dangerous fall of the cat's blood pressure if injected intravenously. A similar but long-lasting hypotension is observed after intravenous administration of yohimbine and of Regitine. Atropine has also a hypotensive action in ether anaesthesia (Bussell, 1940). The same is true for quinine and quinidine. Sollmann (1945) states that this brief fall of blood pressure is a constant characteristic of the intravenous injection of quinidine or quinine and occurs " apparently quite independent of the cardiac effect " and possibly as an " anaphylactoid phenomenon." Our findings suggest that this hypotensive property, common to the stronger adrenaline inhibitors in Table I, is due to their anti-adrenaline action on the peripheral blood vessels.

Comparison of the reversibility of the anti-adrenaline effect.—From the foregoing results it was clear that anti-adrenaline compounds are not always those which inhibit adrenaline in the lowest concentration. Concentration alone does not suffice to distinguish anti-adrenaline from antihistamine compounds. If the specific inhibition is due to a more stable combination of the antagonistic agent with the adrenaline receptors, then this should become obvious by a slower return to normal, when Locke's solution only is perfused again. Therefore it seemed that the time of elimination might indicate the pharmacological affinity of a competing substance for the receptor group better than the concentration.

The speed of recovery after stopping the perfusion of an anti-adrenaline substance was found to depend on the concentration applied. When a high concentration was used, sometimes many hours were required for the elimination at a constant outflow of about 3 ml./min. Therefore the reversibility of the inhibition produced by different substances was compared when the degree of " saturation " of the adrenaline receptors was the same, that is to say, when equally effective concentrations of the different inhibitors had been used. Thus the time of recovery was investigated for the equally effective concentrations given in Table I. The effect of " ergotoxine ethanesulphonate " when examined in a concentration of 0.02 μg. per ml. (which is 1 in 50 million) persisted for about 100 min. after the perfusion of Locke's solution only. The action of dibenamine was found to be almost irreversible even after 20 hr. The action of yohimbine was prolonged for 2–3 hr., and the action of Regitine for about 1 hr. For the other compounds, however, even for the more potent antihistamine substances, a complete restoration of the sensitivity to adrenaline was obtained after 15–45 min. On the whole, therefore, the anti-adrenaline substances persisted in their effect longer than the antihistamine substances as shown in Fig. 2; however, F933 and tolazoline were exceptions, since their effect was removed by perfusion with Locke's solution after 20–45 min.

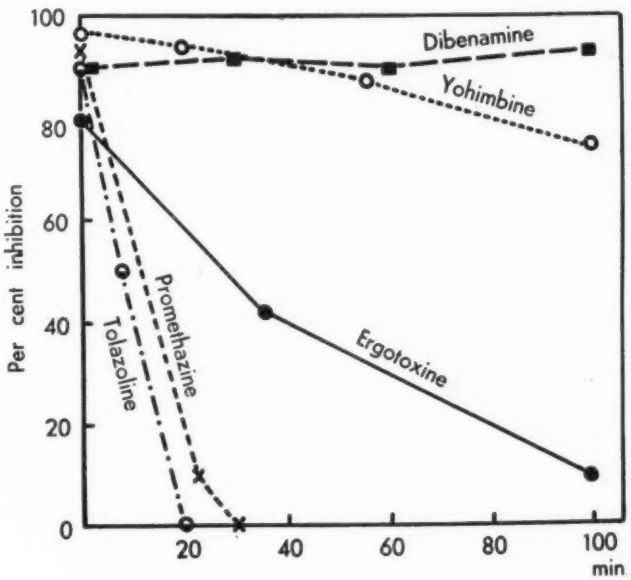

FIG. 2.—The ordinate represents the percentage of inhibition of the constrictor action of adrenaline. The abscissa is time in min. after the change from perfusion with the inhibiting substance back to Locke's solution only. Tolazoline (Priscol) was used as M/32,000. Promethazine was used in M/640,000. Yohimbine was used as M/320,000. Ergotoxine ethanesulphonate was used as 0.02 μg./ml. Dibenamine was used in M/4 million.

Comparison of the affinities for different receptors.—Histamine also produces constriction in the rabbit's ear vessels. This constriction can be abolished by antihistamine compounds, and by other substances such as atropine, pethidine, procaine, and quinidine; thus on rabbit ears it is very easy to determine whether a perfused inhibitor has a greater action against equiconstrictor doses of adrenaline or histamine.

When Regitine was used in a concentration of M/3 million, the equiconstrictor doses were 0.02 μg. adrenaline and 2.0 μg. histamine (Fig. 3). After perfusion with Regitine for 30 min., the effect of adrenaline was reversed while the effect of histamine was greatly reduced. When this was followed by perfusion for 10 min. with Locke's

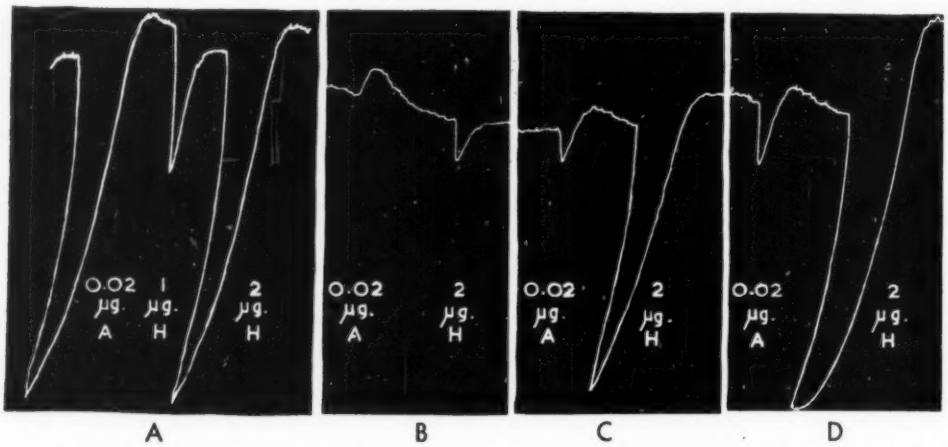

FIG. 3.—To demonstrate the slow disappearance of the anti-adrenaline action and the quick reversibility of the antihistamine action of Regitine. A. Adrenaline (0.02 μg.) and histamine (2 μg.) are equally constrictor. B. Reversal of the constrictor effect of adrenaline by perfusion of Locke's solution containing Regitine (M/3 million) for 30 min. The action of histamine is greatly inhibited. C. After removal of the Regitine from the perfusion fluid for 10 min., adrenaline causes a small vasoconstriction, while the sensitivity of the vessels for histamine has almost recovered to normal. D. Full restoration of the sensitivity to histamine by washing for 25 min. The adrenaline effect remains still very reduced.

solution only, the effect of adrenaline was a very small constriction, while the effect of histamine had returned to more than half the original effect. After perfusion for 25 min. with Locke's solution only, the adrenaline effect was still very small, while the histamine effect was fully restored.

When yohimbine was used, the effects of adrenaline and histamine were equally inhibited, but when yohimbine was washed out the histamine effect returned to normal, whereas the response to adrenaline did not recover for 2–3 hr.

In Fig. 4 it will be seen that yohimbine (M/320,000) reduced the constrictor actions of both adrenaline and of histamine to 1/10th. After removing the yohimbine from the perfusion fluid, the original constrictor effect of histamine returned in 50 min., but the effect of adrenaline remained reduced.

When dibenamine was used the concentrations which sufficed to reduce the constrictor action of adrenaline to 1/10th reduced the constrictor action of histamine even more. Three experiments were carried out in which the effect of dibenamine on the constrictor actions of adrenaline and histamine were compared, and in two of these, after removal of the dibenamine, the histamine constriction began to return

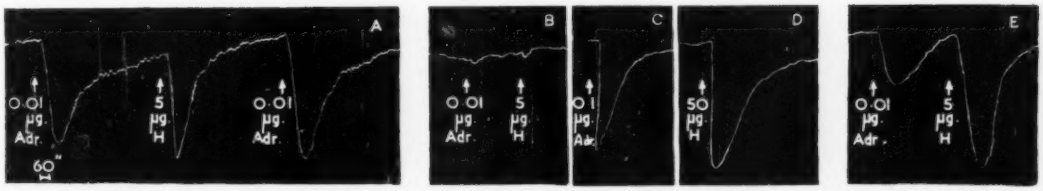

FIG. 4.—The different reversibility of the anti-adrenaline and antihistamine effect of yohimbine. A. Adrenaline (0.01 µg.) and histamine acid phosphate (5 µg.) are equally constrictor. B. The response to these test doses of adrenaline and histamine are abolished by perfusion of yohimbine HCl (M/320,000). C, D. Doses 10 times greater (0.1 µg. adrenaline, 50 µg. histamine) have nearly the same effect as the initial test doses before the perfusion of yohimbine. This indicates that yohimbine reduces the action of adrenaline and histamine to about 1/10th. E. After 50 min. of washing with Locke's solution the full sensitivity to histamine returns whereas the response to adrenaline is still reduced.

before the adrenaline constriction, though the histamine constriction remained depressed. In the third experiment, both the histamine and the adrenaline effects were similarly reduced for 2 hr. after the dibenamine had been washed out.

When F933 was used in a concentration of M/250,000 only the effect of adrenaline was reduced; the action of histamine was not inhibited.

On the other hand, the antihistamine compounds were much more effective against histamine than against adrenaline, in spite of their powerful anti-adrenaline action. Concentrations one-tenth of those required to reduce the effect of adrenaline to 1/10th blocked the histamine receptors completely. When the perfusion was stopped, in contrast to the quick recovery of the adrenaline sensitivity, the histamine effect remained greatly reduced sometimes for many hours as shown in Table III.

TABLE III

STRENGTH AND DURATION OF THE ANTIHISTAMINE EFFECTS OF SOME ANTIHISTAMINE COMPOUNDS ON PERFUSED RABBIT'S EARS, COMPARED WITH THEIR ANTI-ADRENALINE ACTIVITIES

The test doses injected were 0.005 µg.–0.01 µg. adrenaline and equally constrictor doses of histamine acid phosphate

Substance	Mol. conc.	Effect against adrenaline			Effect against histamine		
		Inhibition %	Recovery %	Time of washing (min.)	Inhibition %	Recovery %	Time of washing (min.)
Tripelennamine..	M/64,000	90	100	25	100	5–10	100
	M/640,000	0	—	—	100	100	50
Mepyramine ..	M/32,000	90	100	20	100	0	600
	M/320,000	0	—	—	100	10–20	600
Promethazine ..	M/640,000	ca. 90	100	25	100	0	360
	M/6,400,000	Slightly reduced	—	—	100	25–50	360

DISCUSSION

Previous work has shown that the constrictor action of adrenaline on the vessels of the rabbit ear is not only reduced or reversed by substances well known to depress or reverse the action of adrenaline on the blood pressure, but also by other sub-

stances, some of which are antihistamine compounds and some local anaesthetics, etc. A study has now been made to see what differences there are between these different classes of compounds. The concentration of each compound sufficient to reduce the constrictor action of adrenaline to 1/10th was first determined, as it seemed likely that substances commonly regarded as adrenaline inhibitors would act in a much lower concentration than the others. To some extent this was found to be true; thus the substances acting in the lowest concentration were " ergotoxine " and ergotamine, Regitine, and dibenamine. On the other hand, some antihistamine compounds acted in lower concentration than yohimbine and tolazoline (Priscol). Thus concentration by itself was not a sufficient guide to distinguish substances as anti-adrenaline compounds.

An attempt was then made to measure the reversibility of the anti-adrenaline effect. After the constrictor action of adrenaline had been reduced to 1/10th of the original by perfusing the compound for 30 min., the perfusion was continued with Locke's solution alone, and the time of recovery of the adrenaline effect was determined. The effect of dibenamine was found to be almost irreversible. The effect of yohimbine persisted for 2–3 hr. or more, while the effects of " ergotoxine " and of Regitine persisted for 1–2 hr. In contrast with these substances, the action of all the other substances was completely reversed in periods from 15–45 min. This group of substances included F933 and tolazoline (Priscol). On the whole, it appeared that prolonged attachment to the adrenaline receptors was characteristic of the well-known anti-adrenaline compounds.

Finally, the anti-adrenaline action of many of these compounds was compared with their antihistamine action, comparison being made of the effective concentrations and of the rates of elimination. F933 did not modify the constrictor action of histamine in concentrations which reduced the constrictor action of adrenaline to 1/10th. Regitine reduced the action of histamine in a concentration which reversed the action of adrenaline. Yohimbine reduced the constrictor action of the two substances equally. Dibenamine also seemed to act equally against histamine and adrenaline. Thus the behaviour of these substances towards adrenaline and histamine differed according to the substance. With Regitine, yohimbine, and dibenamine, however, the constrictor effect of histamine returned more rapidly than that of adrenaline after the antagonist had been washed out.

The reverse was true of the antihistamine substances. They abolished the constrictor action of histamine in lower concentrations than that of adrenaline, and after they had been washed out their effect on the histamine response lasted longer. " Specificity " of an inhibiting substance is obviously due to a preference for a certain type of receptor. This preference may show itself in two ways: (*a*) By the concentration in which one type of receptor is blocked, while other receptors are not affected. This type of inhibition may be quickly reversed; F933 is an example. (*b*) By a specially close and stable combination with one type of receptor, whereas the attachment to other receptors is more labile. Yohimbine, Regitine, and dibenamine are examples.

This second type of specific inhibition is reversed slowly. It may be missed if the effective concentrations only are determined. If injected into an animal such a substance may be expected to attach itself to those receptors only which form a stable combination with it.

Highly specific inhibitors may, however, also show a capacity for unspecific blocking at higher concentrations, and a general overlapping may occur; thus, antihistamine compounds block adrenaline and acetylcholine receptors, whereas atropine and other specific acetylcholine antagonists may combine with histamine and adrenaline receptors. Under these conditions two fundamentally different categories can be distinguished on isolated organs: substances which may be called agonists, such as adrenaline, acetylcholine, and histamine; and their common antagonists, antihistamine and anti-adrenaline drugs, acetylcholine antagonists, and simple local anaesthetics. It must be emphasized that this classification is not only the result of pharmacological findings, but also of physicochemical considerations. All " agonists " or " antagonistic " agents included in this study are nitrogen-containing compounds, with ability to act as cations under physiological conditions and to be adsorbed on negatively charged groups (receptors) of the cell surface. Thus, if the " antagonists " act by competition, a greater affinity of the inhibitory cations for the negative receptors must be assumed. In a recent study, Fleckenstein, Guenther, and Winker (1951) used the artificial collodion membrane in order to measure electrically the adsorption and retention of about sixty nitrogen-containing organic cations. Clear differences between the " agonist " and " antagonist " categories were found. All " agonist " substances (adrenaline and other sympathomimetic compounds, acetylcholine and related drugs, histamine, coniine, nicotine, veratrine) were found to be adsorbed loosely on the negative structures of the membrane and to be washed out again very quickly. On the other hand, the " antagonistic " substances (anti-adrenaline, anti-acetylcholine, and antihistamine compounds as well as quinine, pethidine, and simple local anaesthetics) had a much greater affinity for these negative receptors, and were much less readily washed out; indeed, their adsorption on the collodion membrane was frequently irreversible. Therefore it is suggested that the unspecific action of " antagonists " against all types of loosely adsorbed " agonists " may be a simple physicochemical consequence of differences in adsorbability.

It can also be understood why many basic dyestuffs, well known for their affinity for the collodion membrane (Mond and Hoffmann, 1928; Wilbrandt, 1935), exhibit a powerful anti-acetylcholine and antinicotine effect on the isolated frog rectus (Fleckenstein *et al.*, 1951). Most of these basic dyestuffs were considerably stronger than methylene blue, which was first described by Cook (1926) as an acetylcholine antagonist. Furthermore, some recent results with basic fuchsine on rabbit ears show that a similar activity can be exerted against adrenaline. Strychnine is another substance which is strongly adsorbed on the collodion membrane and is an adrenaline inhibitor as well (see Table I).

Whatever the significance of these physicochemical results may be, adrenaline, acetylcholine, and histamine are, however, in some respect related drugs, having common antagonists on certain isolated organs. This conception, put forward first by Burn and co-workers, is given further support by the new results presented.

Summary

1. The anti-adrenaline potencies of various substances, including anti-adrenaline and antihistamine compounds, simple local anaesthetics, and substances like atropine, pethidine, quinine, quinidine, tolazoline (Priscol), and strychnine, have been

investigated in a comparative study on the perfused vessels of the rabbit's ear. Stephenson's outflow recorder was used, and proved to be a satisfactory method of registering very small changes of vascular tone.

2. Ergot alkaloids (" ergotoxine ethanesulphonate," ergotamine tartrate), Regitine and dibenamine, which are generally considered as " specific " inhibitors of adrenaline, were active at very low concentrations. Some antihistamine compounds also exhibit a high anti-adrenaline action: promethazine (Phenergan) and phenindamine (Thephorin) surpass the anti-adrenaline substances yohimbine and F933, and most antihistamine compounds were stronger than tolazoline (Priscol).

3. When the sensitivity of the vessels to adrenaline has been abolished by ergot alkaloids, Regitine or yohimbine, a slow return to normal is seen when pure Locke's solution is perfused afterwards. The action of dibenamine is almost irreversible. The effect of " unspecific " adrenaline antagonists, such as antihistamine and other compounds, is quickly abolished when the antagonist is washed out. Tolazoline (Priscol) and F933 do not differ in this respect from unspecific adrenaline inhibitors.

4. Many of the compounds tested for their anti-adrenaline potency inhibit the constrictor action of histamine too. This is true for yohimbine, Regitine, tolazoline, and dibenamine, and, as already pointed out by Burn and Dutta, for atropine, pethidine, quinidine, and simple local anaesthetics. Antihistamine compounds block the histamine receptors in extremely small concentrations which are ineffective against adrenaline.

5. Yohimbine, Regitine, and dibenamine, which are regarded as specific adrenaline inhibitors, antagonize the vascular constriction produced by adrenaline and histamine to a similar extent, but when they are washed out, the response to adrenaline remains reduced for a much longer time than the response to histamine. Antihistamine compounds, on the contrary, block the histamine effect for a long time and allow a quick recovery of the sensitivity to adrenaline.

6. These observations justify the assumption that " specific " inhibition is due to a preference for a certain type of receptor. This preference may be obvious (*a*) by the low concentration at which one type of receptor is blocked while other receptors are not affected, or (*b*) by a specially close and stable combination with one type of receptor, whereas the attachment to other receptors is more labile.

7. It is suggested that the action of unspecific antagonists of adrenaline or histamine may be due, if they are present in sufficiently high concentration, to their being more firmly bound by adsorptive forces to the negatively charged structures of the cell membrane than either agonist is.

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ACETYLCHOLINESTERASE ACTIVITY OF *SCHISTOSOMA MANSONI**

BY

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Bülbring, Lourie, and Pardoe (1949) demonstrated the presence of acetylcholine in *Trypanosoma rhodesiense* and its absence in the erythrocytic forms of malaria parasites. They suggested that acetylcholine might play a role connected with the motility of parasites. Should this hypothesis be correct, mechanisms for the hydrolysis of acetylcholine should be present not only in protozoan, but also in metazoan parasites which have a well-developed muscular apparatus. Several helminths contain esterase activity since fatty acid esters of glycerol as well as acetylcholine are hydrolysed by homogenates and extracts of these organisms (Bacq and Oury, 1937; Penoit-De Cooman, 1940; Artemov and Lurje, 1941; Penoit-De Cooman and Van Grembergen, 1942). However, whether helminths contain a specific acetylcholinesterase has not been determined previously. Therefore, this problem was investigated in the trematode, *Schistosoma mansoni*. It was found that this parasite contains an acetylcholinesterase which, in many respects, is similar to that occurring in the nervous tissue of vertebrates.

Methods and Materials

Adult schistosomes were obtained as in previous studies (Bueding, 1950; Bueding and Peters, 1951). Cholinesterase activity of worm homogenates was determined according to Nachmansohn and Rothenberg (1945) in an atmosphere of 5 per cent CO_2 in nitrogen. After temperature equilibration (38° C.) the substrate was tipped from the sidearm into the main compartment. Enzymatic activity which remained constant for 20 minutes was expressed in μl. CO_2 liberated per mg. of worms (dry weight) during the first 15 minutes. CO_2 production by the reaction mixture without homogenate, and by the homogenate without substrate, was measured simultaneously and appropriate corrections for these " blank " values were made in the calculation of the esterase activity of the worms. Unless specified otherwise, the concentration of the substrate in the reaction mixture was 4×10^{-2}M. In order to save material and in order to increase the sensitivity of the assay procedure, Warburg flasks were used whose total volume did not exceed 5 ml.

Results

It has been demonstrated that acetylcholine esterase does not split butyrylcholine in concentrations at which a high rate of hydrolysis of acetylcholine is observed,

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whereas other choline-splitting esterases, e.g., serum choline esterase, hydrolyse butyrylcholine at a more rapid rate than acetylcholine (Nachmansohn and Rothenberg, 1945; Augustinsson and Nachmansohn, 1949). As illustrated in Table I, the rate of hydrolysis of acetylcholine by schistosome homogenates was three to four and one-half times greater than that of butyrylcholine. This observation suggested that the schistosomes contain acetylcholinesterase and, in addition, a weaker non-specific cholinesterase. Acetylcholinesterase activity was the same in male and in female schistosomes (Table I) and remained constant for at least several months when the intact organisms were stored in the frozen state at −30° C. Acetylthiocholine and butyrylthiocholine were hydrolysed by schistosome homogenates at a somewhat faster rate than acetylcholine and butyrylcholine respectively.

Homogenates of the muscle of *Ascaris lumbricoides* and of the filarial worm, *Litomosoides carinii*, hydrolysed acetylcholine at a more rapid rate than butyrylcholine (Table I). These observations indicate the presence of acetylcholinesterase in two other helminths. However, it should be noted that the acetylcholinesterase activity of *Ascaris* muscle was considerably lower than that of schistosomes.

TABLE I

HYDROLYSIS OF ACETYLCHOLINE AND OF BUTYRYLCHOLINE BY HOMOGENATES OF HELMINTHS

Substrate concentration, 0.04M

Homogenate of	μl. CO_2/mg. during first 15 min.					
	Acetylcholine Exp. No.			Butyrylcholine Exp. No.		
	1	2	3	1	2	3
Male schistosomes	5.8	6.45	6.1	1.2	2.0	1.6
Female schistosomes	5.7	6.7	6.1	1.3	1.9	1.75
Mixture of male and female schistosomes	5.8	6.6	6.0	1.3	2.2	1.7
Filariae	15.1	15.5	16.3	6.7	6.2	6.5
Ascaris muscle	0.37	0.52	0.36	0	0.08	0

That the hydrolysis of acetylcholine by *Schistosoma mansoni* was catalysed by a specific acetylcholinesterase, which could be separated from the enzyme responsible for the hydrolysis of butyrylcholine, was shown by centrifuging the homogenate at 18,000 r.p.m. for 60 minutes (0°–2° C.) and homogenizing the residue in a solution containing 0.1 M-NaCl and 0.025 M-$NaHCO_3$. Acetylcholinesterase activity was present in this preparation, but butyrylcholine was not hydrolysed by it (Table II). When this preparation was centrifuged at 3,500 r.p.m. for 15 minutes (0°–2° C.), little or no hydrolysis of acetylcholine occurred in the supernatant. The solubility of the schistosome acetylcholinesterase could be increased considerably by ultrasonic treatment of the homogenized residue (5 ml.) in a 9 KC Raython sonic oscillator for 2½ minutes at 1° C. (Table II).

The effect of substrate concentrations on the acetylcholinesterase activity of *S. mansoni* is recorded in Fig. 1. The optimal concentration was found to be 0.04 M. Higher concentrations of acetylcholine had an inhibitory effect. By contrast, the rate of hydrolysis of triacetin increased with the substrate concentration, but even

TABLE II

ESTERASE ACTIVITY OF FRACTIONATED HOMOGENATES OF *Schistosoma mansoni*

Substrate concentration, 0.04M

Fraction	µl. CO_2/mg. during first 15 min.			
	Acetylcholine Exp. No.		Butyrylcholine Exp. No.	
	1	2	1	2
Untreated homogenate	6.7	5.9	1.8	1.4
Supernatant obtained by centrifugation of homogenate at 18,000 r.p.m. (1 hr.)	2.1	2.2	1.4	1.2
Residue obtained by centrifugation of homogenate at 18,000 r.p.m. (1 hr.)	4.5	3.6	0.4	0
Supernatant obtained by centrifugation at 3,500 r.p.m. (15 min.) of suspended residue	0.7	0	0	—
Supernatant obtained by centrifugation at 3,500 r.p.m. (15 min.) of suspended residue subjected to *ultrasonic vibration*	4.35	3.3	0	—

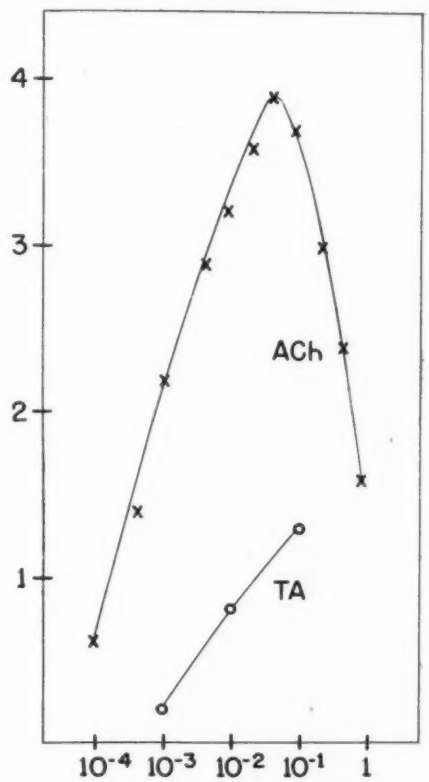

FIG. 1.—Effect of substrate concentration on acetylcholinesterase activity of *Schistosoma mansoni*. A suspension of the residue obtained by centrifugation of a schistosome homogenate at 18,000 r.p.m. (1 hr., 1° C.) was used as a source of enzyme. Abscissae: log of molar substrate concentration. Ordinates: esterase activity (µl. CO_2/mg.). ACh = Acetylcholine. TA = Triacetin.

at the highest concentration which was tested (0.1 M), triacetin was hydrolysed at a rate which was considerably lower than that observed with optimal concentrations of acetylcholine.

As with vertebrate tissues, the activity of acetylcholinesterase of schistosomes was inhibited by physostigmine and neostigmine. Complete inhibition was observed at molar concentrations of 1×10^{-5} with either of these two compounds.

Discussion

Acetylcholinesterase of *Schistosoma mansoni* is similar to the acetylcholinesterases of other species in the following respects: (1) In concentrations at which a high rate of hydrolysis of acetylcholine is observed butyrylcholine is not hydrolysed. (2) Reduction below as well as an increase above the optimal concentration of acetylcholine results in a decrease of the enzymatic activity. (3) Triacetin is hydrolysed at a slow rate. These properties distinguish acetylcholinesterase from other choline ester-splitting enzymes (Augustinsson and Nachmansohn, 1949). The only observed difference between the acetylcholinesterase of *S. mansoni* and that obtained from other tissues consists in the fact that the optimal substrate concentration for the schistosome enzyme is higher. The presence in schistosomes of a specific acetylcholinesterase, similar in properties and in concentration to that of central nervous tissue, suggests that acetylcholine may play a functional role in this parasite. This hypothesis is supported by an unpublished finding of Nachmansohn (personal communication) that acetone powders of schistosomes contain choline acetylase, i.e., an enzyme which catalyses the synthesis of acetylcholine from choline and acetate in the presence of adenosinetriphosphate and coenzyme A (Nachmansohn, Hestrin, and Varipaieff, 1949), at a rate of 135 to 150 μg. acetylcholine per g. per hour. Bülbring *et al.* (1949) have correlated the motor activity of parasitic protozoa with the presence and the production of acetylcholine. The presence of acetylcholinesterase and of choline acetylase in *Schistosoma mansoni* suggests that this applies also to parasitic metazoa, particularly because acetylcholinesterase activity has been detected in two other helminths, the filarial nematode, *L. carinii*, and in *Ascaris lumbricoides*. Baldwin and Moyle (1949) have shown that eserine does not sensitize *Ascaris* muscle to the effect of low concentrations of acetylcholine. It should be noted that the acetylcholinesterase activity of this tissue is considerably lower than in the schistosomes and the filariae.

Summary

The trematode, *Schistosoma mansoni*, contains a specific acetylcholinesterase. Its concentration in the parasite is of the same order of magnitude as that of mammalian brain. Some properties of this enzyme are described and compared with acetylcholinesterases from other species.

The author is indebted to Dr. David Nachmansohn for his most valuable advice and suggestions.

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THE QUANTITATIVE ASSAY OF VASOPRESSIN

BY

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There are three animal preparations which may be used for the biological assay of pituitary pressor potency: the spinal cat (Dale and Laidlaw, 1912; Hogben, Schlapp, and Macdonald, 1924), the anaesthetized dog (Hamilton and Rowe, 1916; Kamm, Aldrich, Grotte, Rowe, and Bugbee, 1928), and the anaesthetized rat (Landgrebe, Macaulay, and Waring, 1946); of these, the rat preparation is the most economical, the most sensitive, and the most reliable.

In a previous report (Dekanski, 1951) dealing with the preparation and qualitative assay of a pressor fraction from normal urine, N: N-dibenzyl-β-chloroethylamine (" Dibenamine ") was injected into the rat preparation in order to inhibit the action of certain pressor substances known to be present in fresh urine. Dibenamine was found to have a potent and prolonged action in abolishing the usual pressor effects of adrenaline, noradrenaline, hydroxytyramine, nicotine, and piperidine. On the other hand, the rise in blood pressure produced by pitressin or by the urinary pressor fraction was not abolished. The results of these experiments indicated that a quantitative assay of the pressor potency of pituitary extracts might be readily obtained by means of a comparatively simple rat's blood pressure preparation into which dibenamine had been injected.

In the rat preparation described by Landgrebe *et al.* (1946) for pressor assays of pituitary extracts, the vagi and associated sympathetics are severed and the posterior cord pithed. Neither is, in fact, necessary if the rat preparation is treated with dibenamine. Subcutaneous urethane anaesthesia was found to be as efficient as that by " Dial " followed by urethane both injected intraperitoneally. The modified rat's blood pressure method, appears to be more satisfactory and more specific than any other method for the assay of pressor activity of pituitary preparations.

METHODS

Technique.—A male albino rat weighing about 300 g. is anaesthetized with urethane (175 mg. per 100 g. body weight) injected subcutaneously. After 45 to 60 min. the rat is tied to the operating table by its hind legs, the front legs not being secured. The trachea is cannulated with a short glass or polythene tube of about 2.5 mm. external diameter, and one carotid artery dissected ready for cannulation. The femoral vein close to the inguinal ligament is cannulated with a special polythene cannula. The abdominal muscles are retracted to expose the inguinal ligament. The superficial pudendal vein is retracted to one side and the femoral vein dissected towards the inguinal ligament from the corresponding artery. The other deep branch reaching the femoral vein has to be found and tied off, otherwise bleeding may occur during cannulation. A short polythene cannula

is tied into the femoral vein by two ligatures and joined by a short piece of rubber tubing to a 1 c.c. burette with an attached thistle funnel containing warm normal saline. Finally, the cannula is fixed firmly to the thigh. The burette is attached to the operating table to avoid accidental disconnection. Heparin (200 u. per 100 g. body weight) is injected through the venous cannula and washed in with saline. The carotid cannula is tied in and connected with a special mercury manometer (Crawford and Outschoorn, 1951) of about 2 to 3 mm. internal diameter by a column of normal saline. Both the venous and the carotid cannulae are of 1 mm. external diameter. The central and peripheral nervous system, including both vagi and associated sympathetics, is left intact. No artificial respiration is necessary. All solutions, filtered if necessary and warmed to body temperature, are injected through the venous cannula by means of a 1 c.c. tuberculin syringe and are washed in with 0.2 c.c. normal saline from the burette. Care should be taken that no air is injected, as the smallest air bubble may be fatal to the animal.

At first the preparation does not maintain a constant basal pressure, but 20–30 min. after the intravenous injections of dibenamine the blood pressure establishes itself on a new basal level of about 50 mm. Hg and remains constant for at least 8 hours. Dibenamine, 100 μg. per 100 g. body weight per dose, is injected twice at an interval of 10 min. Twenty minutes after the first injection of dibenamine the volume effect due to the injection of 0.3–0.5 c.c. normal saline should be significantly reduced; if not, a third dose of dibenamine is usually sufficient to minimize or even to abolish the blood pressure response to the injection of saline, and to produce a constant basal pressure.

During this preparatory period a rough estimate of the potency of some unknown solution may be obtained, by matching it with two dose-levels, about 6 mu. and 10 mu. of the International Pituitary Posterior Lobe Standard (100 mu./c.c. normal saline, freshly made each time). It was found (Landgrebe *et al.*, 1946) that even a large dose of the unknown solution can be injected without affecting the response to the succeeding smaller doses.

In each assay four doses are used, two of the standard and two of the unknowns, the ratio of high to low dose being the same (usually 3 to 2) for both the standard and the unknown. Each dose is injected every 6 to 10 min. once in each group of four, and there are 6 groups in all.

Drugs used:

Subcutaneously: Urethane (B.D.H.) 25 g. per 100 c.c. solution in distilled water—0.7 c.c. per 100 g. body weight.

Intravenously: Heparin B.P. (Boots) sterile, 200 u. per 100 g. body weight dissolved in 1 to 2 c.c. normal saline.

Dibenamine HCl or N: N-dibenzyl-β-chloroethylamine hydrochloride (Smith, Kline, and French)—1 mg./c.c. solution in faintly acid saline prepared by dissolving 5 mg. dibenamine in 0.1 c.c. 95 per cent ethanol made acid (approx. 0.05 N) with conc. H_2SO_4 and then diluted up to 5 c.c. with normal saline.

Various commercial pituitary preparations.

Results and Discussion

Fig. 1 demonstrates the blood pressure responses to increasing doses of pituitary standard. Fig. 2, which has been constructed from Fig. 1, shows the log dose-response regression line relating response to log dose of pituitary. A straight log dose-response regression line could always be obtained by the method used. The preparation treated with dibenamine maintains a constant basal pressure and readily discriminates between doses, differing by not more than 15–20 per cent. The

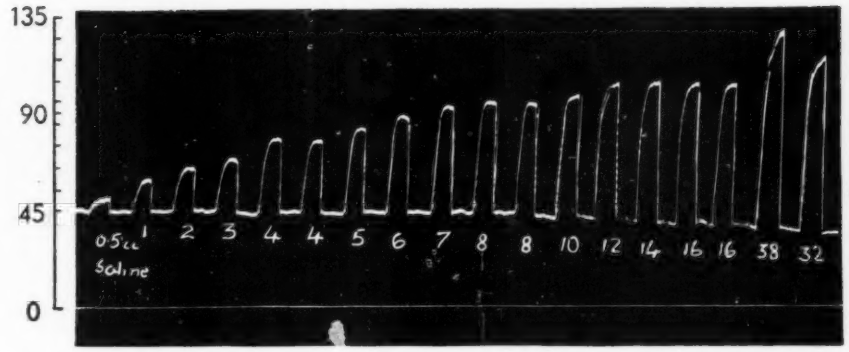

FIG. 1.—Rat's blood pressure responses to different doses of pituitary standard ranging from 1 to 38 milliunits (100 mu./c.c.). From these results a log dose-response graph has been constructed in Fig. 2. Weight of rat 270 g.

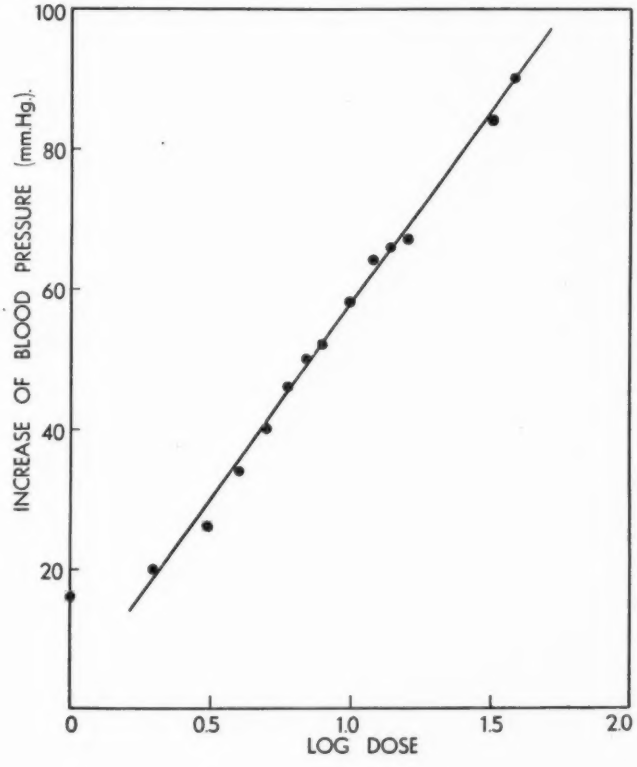

FIG. 2.—Log dose-response regression line relating response to log dose of pituitary standard, based on results shown in Fig. 1.

injections of small doses (4 to 12 milliunits) can be made every 6 to 10 min. without tolerance developing, and the responses are satisfactory for about 40 injections.

With doses of 15 to 100 milliunits the blood pressure takes at least 15 min. to return to the basic level and the response usually becomes erratic.

The results of a pressor assay of the pituitary are given in Fig. 3, from which Tables I and II and Fig. 4 have been constructed. Fig. 4 represents the simple

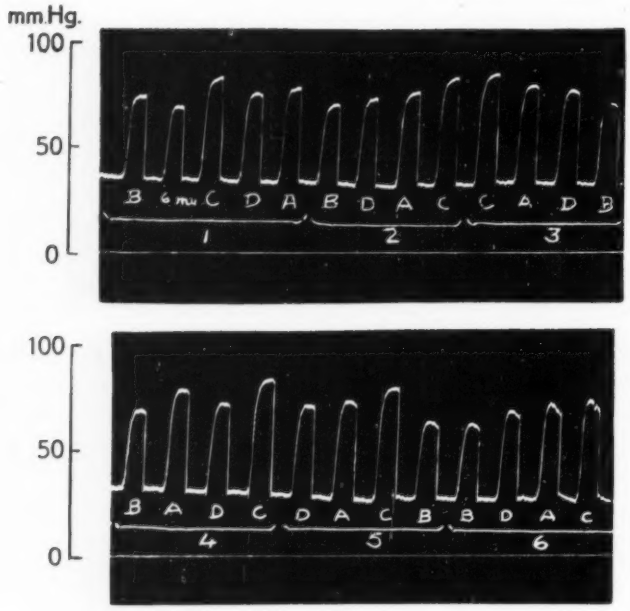

FIG. 3.—The pressor assay of pituitary standard. The record shows 24 responses of a rat blood pressure. The increases of blood pressure are responses to four different doses of pituitary standard A, B, C, and D, each of which was injected once in each group of four. There are six groups in all. A = 10 mu., B = 7 mu., C = 12.5 mu., and D = 8.75 mu. A and B were treated as "standard." C and D were treated as "unknown." D : C = B : A = 7 : 10. Estimates of unknown : standard = 1.251. True value unknown : standard = $\frac{8.75}{7}$ = 1.250. A dose was injected intravenously every 8 min. and washed in with 0.2 c.c. normal saline. Weight of rat 300 g. Total dose of dibenamine 900 μg. Total experimental time about six hours.

TABLE I

INCREASE OF BLOOD PRESSURE IN MM. HG = Y

	Concentration mu./c.c.	Dose c.c.	Dose mu.	Log dose = x	1	2	3	4	5	6	Sum	Mean
A	100	0.10	10	1.000	46	45	47	48	47	46	279	46.5
B	100	0.07	7	$\bar{1}$.8451	40	38	39	38	38	38	231	38.5
C	125	0.10			50	50	52	54	54	50	310	51.7
D	125	0.07			44	43	45	43	44	42	261	43.5
Group totals					180	176	183	183	183	176	1,081	

TABLE II

Source of variation	Sum of squares	Degrees of freedom	Variance	F	P
1. Groups	15	5	3	2.37	>0.05
2. Standard and unknown ..	155	1	155	118.4	<0.001
3. Regression	392	1	392	309.4	<0.001
4. Deviation from parallelism ..	<1	1	<1		
5. Residual error	19	15	1.267		
Total	581	23			

$$S_M = \sqrt{\frac{4 \times 1.267}{24 \times 52.2}\left(\frac{0.09742}{0.15492} + 1\right)} = \sqrt{0.00010814} = 0.0104$$

For 15 degrees of freedom t = 2.13 (P = 0.05). The fiducial limits for M = $\pm S_M \times t = \pm 0.0104 \times 2.13 = 0.0222$.

Since M = $\bar{1}$.9026, fiducial limits are $\bar{1}$.9248 and $\bar{1}$.8804. The fiducial limits for R = 119.0 and 131.7 mu./c.c.

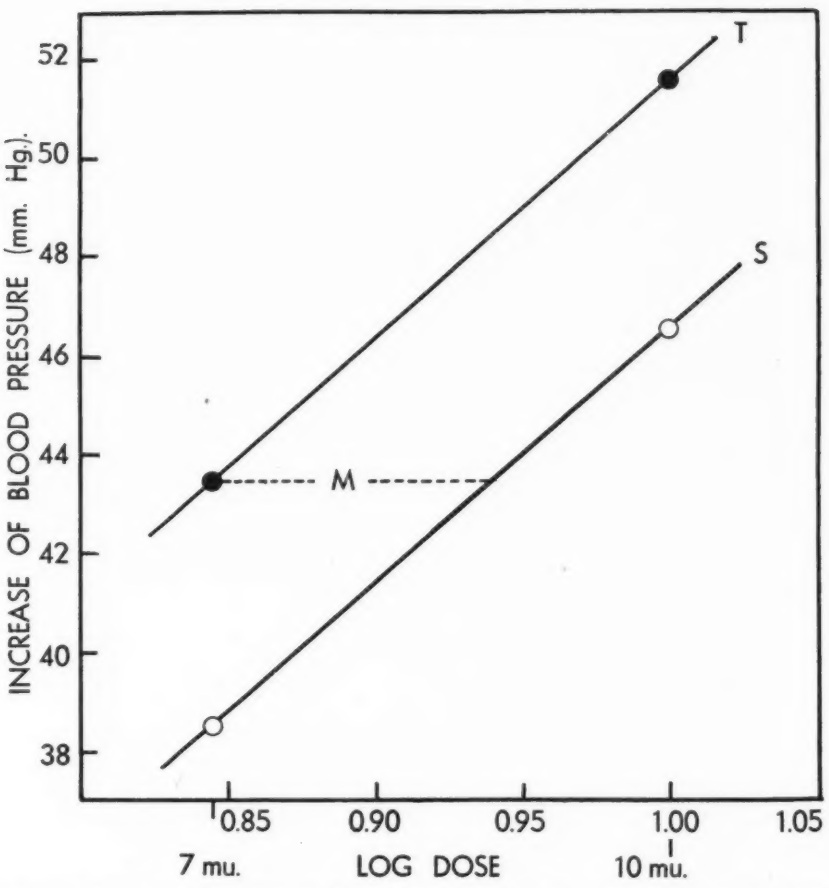

FIG. 4.—Ordinates: mean increase of blood pressure in mm. Hg. Abscissae: Log_{10} dose in milliunits of pituitary standard. White circles are the mean responses for high and low doses of standard, black circles are the mean responses for high and low doses of unknown. S and T are the regression lines relating response to log dose of standard and unknown. The lines were drawn by eye. Graphically $M = \log (0.943 - 0.845) = 0.098$.

$$\therefore \frac{\text{unknown}}{\text{standard}} = \text{antilog } 0.098 = 1.253.$$

graphical measurement of M, the log of the ratio of unknown to standard, and Table II the calculation of the potency ratio and error, by the methods of calculation and analysis of variance given by Schild (1942) and Holton (1948). By calculation the true value of M was found to be $\bar{1}.9026$. Hence R=0.7991 and the potency per c.c.=125.1 mu. The slope b=52.2, and the index of precision $\lambda = Sy/b = 0.0215$.

The method has been found to give satisfactory results in the routine assays of the pressor activity of various commercial pituitary preparations. From 30 such assays the mean λ with standard error was found to be 0.042 ± 0.023.

These results seem to prove that the rat preparation treated with dibenamine is a simple, sensitive, and specific method for the quantitative assay of pressor potency both in pituitary extracts and in commercial pituitary preparations. The rat does not react to the pituitary oxytocic factor in this way. The pressor response

is not interfered with by the possible presence of small amounts of other pressor substances nor by that of small amounts of histamine (Landgrebe *et al.*, 1946) which is a common contaminant of the pituitary extracts.

Summary

1. A four-point procedure was applied to the pressor assay of posterior pituitary extract and various pituitary commercial preparations.

2. A simple rat's blood pressure preparation treated with dibenamine was found to be the most sensitive and the most specific test preparation.

3. The mean index of precision with standard error (30 values of λ) was found to be 0.042 ± 0.023.

I wish to thank Prof. J. H. Gaddum for his constant interest and advice in this work.

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OBSERVATIONS ON THE PASSAGE OF A COLLOID FROM CEREBROSPINAL FLUID TO BLOOD AND TISSUES

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The permeability of the membranes separating blood and cerebrospinal fluid has been the subject of many investigations. In a common type of experiment, introduction of a substance into one of these fluids is followed, after appropriate intervals, by a determination of its concentration in the other. When an injection is made into the subarachnoid space, it is clear that the results of blood analysis will depend not only upon the rate of passage of the test material through the membranes, but also upon the rate of its removal from the blood. Injection of substances into the vascular system also involves this factor.

The behaviour of the material in the blood is of particular importance when foreign colloids are studied, since here there is likely to be a slow passage through the membranes associated with a rapid removal from the blood. This communication is a short survey of such a problem.

Colloidal palladium was used in this work ; it was selected because a stable and reasonably homogeneous suspension could be prepared. Colloidal palladium has seldom been referred to in biological literature, but brief references to it have been made by Rebière (1908), Neuberg, Caspari, and Löhe (1912), Duhamel (1919). Courmont and Dufourt (1913), and Goffin and Goffin (1922). The *Extra Pharmacopoeia* (1932) mentions " Pallamine," a colloidal palladium.

MATERIALS

Preparation of the palladium.—Samples of Pd^{109} were obtained from the Harwell pile by irradiation for one week of a number of 300 mg. samples of palladium sponge. In the maximum neutron flux available, the calculated activity was 420 mc./g. $\pm$ 20 per cent at the end of the irradiation period.

Palladium was separated from the silver impurity by one of two methods. The sponge was dissolved in 5 c.c. of warm aqua-regia containing between 16 and 20 mg. $AgNO_3$ to serve as carrier. In the first method the solution was diluted to 0.5 litre with distilled water and the palladium precipitated with dimethyl glyoxime and filtered off. After being washed, the precipitate was dried, when it separated from the filter paper as a thin wafer which was again dissolved in a small volume of aqua-regia. The solution was heated and concentrated hydrochloric acid added drop by drop until nitrogen peroxide ceased to be evolved. The mixture was next evaporated to near-dryness, then diluted to 50 c.c. with distilled water and centrifuged, after which a sample of the solution was

examined microscopically in order to ensure the absence of gross particulate matter; 0.5 N-NaOH was titrated against a small sample of the solution and the end-point determined either with litmus or by the appearance of a persistent brown precipitate with a clear supernatant.

In the second and more rapid method, advantage was taken of the solubility of AgCl in concentrated HCl. The sponge and carrier were dissolved as before and concentrated HCl added to remove the nitric acid. The solution was evaporated almost to dryness and then diluted to 50 c.c. with distilled water. Much of the AgCl was then precipitated and removed by centrifugation. The evaporation and dilution were repeated with further carrier.

Preparation of the colloid.—The method was essentially that of Paal and Amberger (1904). The colloid was dialysed against running tap-water for ten hours. Occasionally a few brown flakes were seen in the colloid, but these were absent after dialysis.

Concentration of colloid.—The colloid was concentrated under reduced pressure, small plugs of glass wool, separated by drilled baffles of perspex, being arranged in the neck of the flask to reduce the frothing frequently attending this operation. The colloid, now some 20 c.c., was centrifuged for one hour at 1,300 g. There was usually about 0.1 c.c. of sludge at the bottom of the cups and this was discarded. The colloid was dense black in bulk, whereas films appeared dark brown, and the final concentration was usually a little less than 1 per cent Pd. Small samples were set aside for gravimetric assay and electron microscopy. Non-sterilized samples stored for 12 months have shown very little precipitate.

Size of particles.—Electron microscopy of a typical colloid showed a distribution of particle diameters as follows: 54%, 200A; 15%, 300A; 15%, 100A; 11%, 400A; 5%, 500A. In those samples which had not been centrifuged a few particles of about 1 μ were seen. In all samples examined there appeared to be a number of particles of less than 100A, but these were too small for adequate resolution by the electron microscope available.

Stability and dialysable fractions.—Aggregates of particles were not seen microscopically in samples of circulating blood and very few after incubation with whole blood, plasma, or cerebrospinal fluid. The colloid was able to pass a cellophane membrane in amounts which were usually below the limits of accurate assay.

Results

Blood transport.—From naked eye and microscopical studies it appeared probable that the colloid was carried mainly in the plasma during the first hour at least. This was supported by a series of blood analyses after intravenous injection of the colloid; it was found that the concentration of the colloid in whole blood agreed closely with that obtained for plasma after the appropriate corrections for packed cell volume had been made.

Toxicity of colloid.—It was frequently noted that the colloid could be given intravenously without marked change in blood pressure or respiratory rhythm. With large doses a change in respiratory rhythm and a fall in blood pressure were seen, but successive similar doses administered at the same rate produced effects which were usually less than those produced by the initial dose (Fig. 1). The changes indicated are not entirely due to the protective agent.

It was found that reasonably steady records of cerebrospinal fluid pressure could be obtained under pentobarbitone anaesthesia (40 mg./kg. intraperitoneally)

and that, after an appropriate volume of cerebrospinal fluid had been allowed to escape, the colloid could be introduced into the subarachnoid space without any subsequent gross fluctuation of the cerebrospinal fluid pressure. Further, the presence of the colloid did not prevent those changes in the cerebrospinal fluid pressure which are produced by the introduction of hypertonic fluids into the stomach.

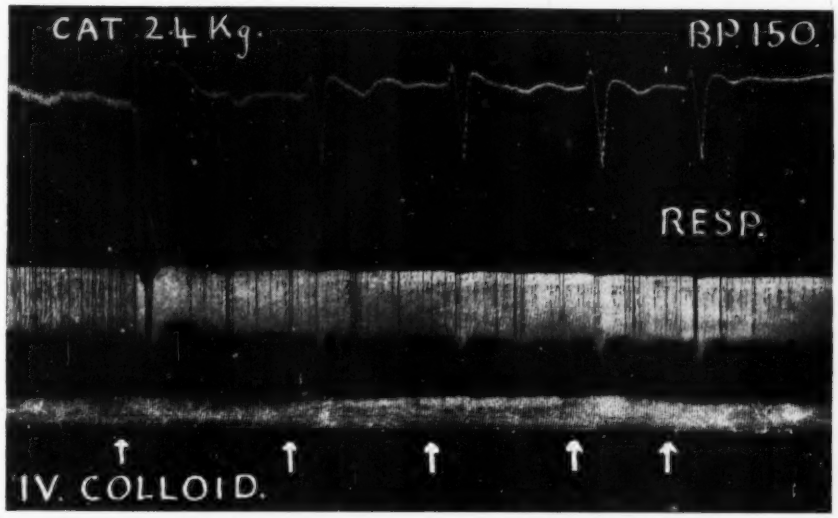

FIG. 1.—The effects upon blood pressure and respiratory rhythm of repeated intravenous injections of 6 c.c. of colloidal palladium containing 6.86 mg. Pd/c.c. The injections were made at the arrows. (Time mark at 5 sec. intervals.)

Assay procedures.—In all estimations of radioactive material a 10 c.c. solution counter was used with an Sc 200A Dynatron scaling unit. The usual corrections were made and also density corrections in the counting rates obtained from those samples which had been digested in sulphuric and nitric acids. Tissues were assayed for Pd after first digesting them in sulphuric and nitric acids in Kjeldahl flasks.

In a typical experiment, designed to investigate the accuracy of the assay procedures, a number of gravimetric assays were performed on a large bulk of radioactive colloid and appropriate dilutions of this digested with liver slices. In ten separate radioactive estimations no value obtained differed from the gravimetric assays by more than 2 per cent, when the concentration of palladium determined by radioactive methods was less than 1 μg./c.c.

Rate of removal of the colloid from the blood.—Cats were used in all animal experiments. They were anaesthetized with pentobarbitone (40 mg./kg. intraperitoneally) and various doses of the colloid were introduced by direct injection into the femoral or saphenous vein. Blood samples were taken from the femoral or carotid artery at intervals during a period of about one hour and assayed for palladium. A total of 10–12 c.c. of blood was taken during each experiment and solid heparin or sodium citrate crystals were used as anticoagulants. The amounts of palladium injected varied between 3.9 μg./kg. and 2.8 mg./kg. The results

(Fig. 2) indicate that over this range the blood level fell, during the first thirty minutes of the experiments, to between 48 per cent and 4 per cent of that observed one minute after the end of the injection.

It was clear that there were considerable differences in the rates of removal of colloidal palladium from the blood, but, on the whole, large doses were cleared more slowly than small doses. The influence of the size of the dose on the rate of its removal from the blood was investigated in two cats by administering a large dose (1.92 mg./kg.) to one of them and a small dose (0.59 mg./kg.) to the other. On the next day, the cat which had received the large dose was given a small one (0.48 mg./kg.), and the cat which had received the small dose was given a large one (2.36 mg./kg.). In each cat the larger dose was removed from the blood more slowly than the smaller dose (Fig. 2), and at the end of the first day the smaller dose

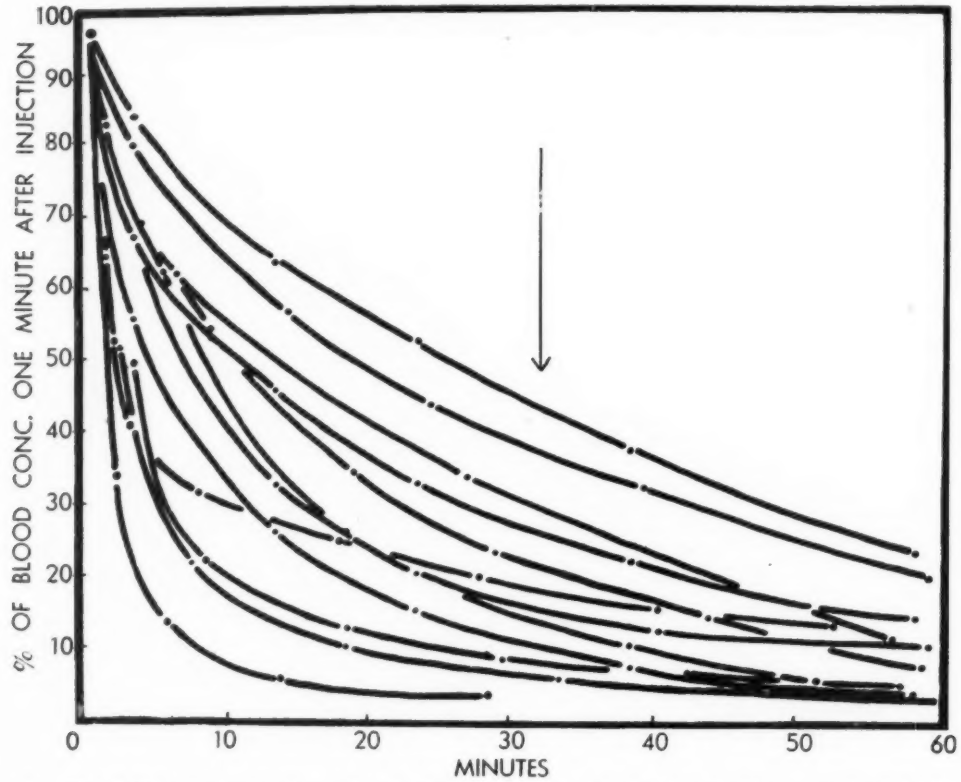

FIG. 2.—The fall in concentration of palladium in the blood of cats after intravenous injection of various doses of colloidal palladium. Following the line of the arrow, the dose (mg./kg.) corresponding to each curve from above downwards is 2.36, 1.92, 2.82, 1.41, 1.34, 2.08, 0.48, 1.03, 0.59, 1.74, 0.22, 0.004.

was practically cleared, while a measurable concentration of the larger dose remained in the blood. The lowest curve (Fig. 2) indicates the limit of assay with the particular technique employed ; with the radioactivity of the palladium at its maximum, the lowest blood concentration which could be satisfactorily determined was 0.0013 μg./c.c.

Fate of intravenous colloid.—Twelve experiments were performed. The animals received intravenous doses of colloid containing between 50.2 and 0.01 mg. of palladium. Samples of arterial blood were taken one minute after injection and at the end of the experiment. The animals were killed by opening the chest, and ten minutes later samples of lung, kidney, liver, spleen, and brain were taken for palladium assay. In the earlier experiments lung was not sampled. As far as possible, specimens were removed from similar sites in a particular organ. Table I indicates that on the whole the fall in blood level was more rapid with smaller doses, though here, as in Fig. 2, there are some anomalous results. Samples of cisternal cerebrospinal fluid were usually obtained by cisternal puncture at the end of the experiment, but the concentration of palladium was usually far below the limits of assay.

TABLE I

DISTRIBUTION OF PALLADIUM IN THE TISSUES OF CATS AFTER INTRAVENOUS INJECTIONS OF COLLOIDAL PALLADIUM

Wt. of cat (kg.)	Total dose (mg.)	Duration of exp. (min.)	Fall of concn. in blood %	% of total dose/g. of tissue					% of dose in urine
				Lung	Spleen	Liver	Kidney	Brain	
3.5	50.2	64	70	1.3	0.41	0.44	0.019	0.0012	1.6
3.6	36.6	75	80	0.52	0.38	0.51	0.018	Trace	0.97
3	24.4	69	85	2.04	0.74	0.41	0.015	0.0016	1.07
2.5	15.2	60	74	2.4	0.76	0.58	0.036	0.0017	0.98
2.2	10.0	62	88	1.7	0.63	0.70	0.022	0.0012	0.82
3.8	10.0	61	87	1.5	0.34	0.45	0.013	0.0006	0.88
1.5	7.6	60	85	3.9	1.9	0.79	0.043	0.0023	0.84
1.9	6.1	65	89	No sample	0.66	0.79	0.019	Trace	1.04
2.5	6.1	73	86	No sample	0.70	0.80	0.019	0.0045	Blood-stained 0.12
3	5.0	64	94	1.05	0.34	0.510	0.034	Trace	1.21
2.5	2.5	60	93	1.2	0.72	0.52	0.048	0.0012	1.3
2.6	0.01	60	96	4.2	0.86	0.90	Trace	Trace	0.58

It appears from these results that the lung contained a larger quantity of palladium per unit weight than any of the other tissues examined. The liver or spleen occupied second place and the total excretion of colloid in the urine was about 1 per cent.

Passage from subarachnoid space to blood.—The vertebral laminae at the root of the tail were removed and a polythene cannula of 1 mm. bore was introduced into the subarachnoid space and tied in position with two spaced silk ligatures. Cerebrospinal fluid was allowed to drip from the cannula and the volume replaced with colloid administered by means of a burette. The burette was adjusted so that the level of colloid within it was never more than 16 cm. above the sacral wound. Colloid was allowed to run in for various periods of time and the volumes introduced varied between 0.7 and 1.9 c.c. The animals were prone throughout the experiment. Blood samples were taken at intervals as before, but it was found that large volumes were required in order to detect the small quantities of circulating colloid. A total of 15–20 c.c. of blood was removed from some animals during the

course of an experiment. Fig. 3 shows the results obtained. There was a considerable variation in the rate of rise of blood concentration and this did not appear to depend either on the total dose administered or upon the volume of colloid introduced.

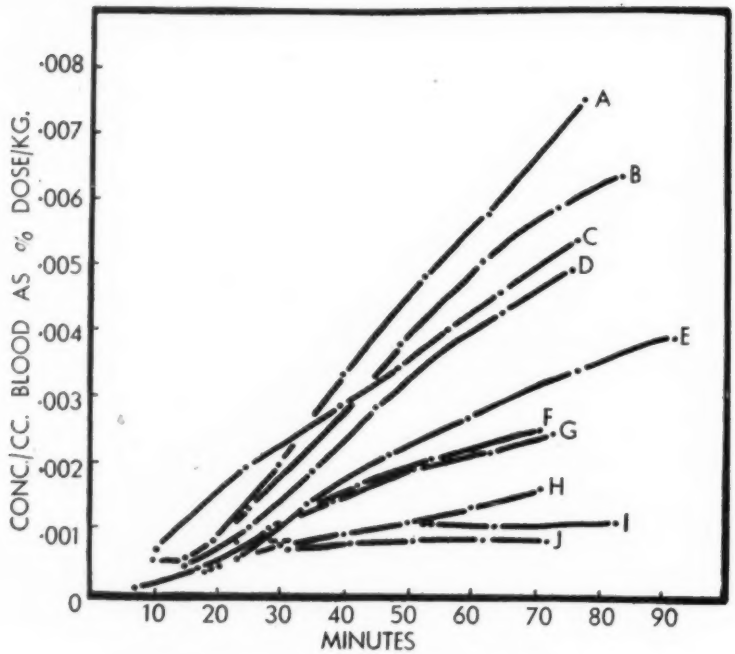

FIG. 3.—The rise in concentration of palladium in the blood after the introduction of various doses of colloidal palladium into the subarachnoid space of cats. Doses in mg./kg. are: A, 1.27; B, 2.82; C, 2.06; D, 1.59; E, 0.74; F, 2.54; G, 3.04; H, 4.36; I, 1.72; J, 1.01.

Tissue distribution after subarachnoid injection.—At the end of the experiments described above, some of the animals were killed and the tissues assayed for palladium. Table II shows that the lungs, as after intravenous injection, contained more palladium per unit weight of tissue than the spleen, liver, or brain. In this series, however, the kidney often contained more than the lung. The brain as a rule

TABLE II

DISTRIBUTION OF PALLADIUM IN THE TISSUES OF CATS AFTER SUBARACHNOID INJECTION OF COLLOIDAL PALLADIUM

Wt. of cat (kg.)	Dose (mg.)	Length of exp. (min.)	Blood concn. at end of exp. μg./c.c.	% of total dose/g. of tissue					% of dose in urine
				Lungs	Spleen	Liver	Kidney	Brain	
3.3	14.4	71	0.074	0.0034	0.0014	0.0014	0.0013	0.0031	0.081
3	7.6	71	0.066	0.0027	0.0014	0.0014	0.0037	Trace	0.093
2.5	7.6	73	0.077	0.0014	No sample	0.00062	0.0042	Trace	0.067
2.5	7.0	84	0.18	0.0036	0.0021	0.0024	0.0074	Trace	0.26
2.6	4.48	84	0.020	0.0091	0.0024	0.0039	0.0013	Trace	0.066
2.8	4.38	78	0.082	0.0023	0.0018	0.0014	0.0050	0.014	0.28
2.7	2.00	92	0.029	0.011	0.0065	0.0042	0.015	Trace	0.056

held such small amounts of palladium that assay could not be satisfactorily performed. In two brain samples, where assay appeared possible, the results obtained were probably due to contamination from the colloid introduced into the spinal subarachnoid space.

Discussion

The present studies indicate that colloidal palladium, with the characteristics described, is rapidly removed from the blood stream of the cat, and that the lungs collect a higher concentration of palladium than any of the other tissues examined, with liver or spleen in the second place. These findings are in accord with those of Drinker and Shaw (1921) and Lund, Shaw, and Drinker (1921), who worked with an acacia-protected colloidal manganese dioxide. These workers also noted that the large initial trapping of colloid by the lungs seemed peculiar to the cat and that even in this animal the lungs subsequently lost much of their colloid to the liver.

The rapid removal of particulate matter from the blood associated with deposition in the liver has been demonstrated for a number of colloids such as yttrium, zirconium, and columbium (Dobson *et al.*, 1949), gold (Sheppard *et al.*, 1951), manganese (Sheppard *et al.*, 1947), chromic phosphate (Jones *et al.*, 1944), and certain brominated dyes (Moore *et al.*, 1943), though with these much dye was also found in the faeces.

Drinker and Shaw (1921) also noted that large doses of colloid usually persisted longer in the blood than small doses, though sometimes a small dose was slowly removed. These anomalies they attributed to some peculiarity in the animal. These observations have been confirmed by the author using colloidal palladium. Dobson *et al.* (1949), from their work with mice, rats, and rabbits, considered that particle size in their colloids governed not only the rate of removal of such material from the blood but also its distribution among the tissues. These conclusions were not supported by Sheppard *et al.* (1951) in their studies with gold sols, though few experiments were reported and the particle size range was apparently small.

In the present research unpredictable variations in the rate of blood clearance were recorded, even with samples of the same preparation of colloid, so that differences in particle size could not be the major factor.

Few of the present results show an " exponential " removal from the blood of the type suggested by Sheppard *et al.* (1947 and 1951) for colloidal gold under " proper " conditions.

It is clear that accurate representation of the rate of removal of particulate matter from the blood presents difficulties. These arise in part from errors made in recording the time of sampling during the period of most rapid removal, and in part from the assumption of a time at which a uniform distribution of the material in the blood occurs.

It is felt that a detailed consideration of the results obtained after subarachnoid injection can serve no useful purpose at the present time, since, of the many variables involved, some have been inadequately controlled or present difficulties in measurement. In this series of experiments it appears that the colloid passed from the subarachnoid space into the blood. The resulting blood concentrations showed

some variation, attaining a maximum of 0.0077 per cent of the injected dose per kg. in 78 minutes.

A comparison of the relative tissue concentrations after intravenous and subarachnoid injections reveals no striking differences except in the kidney. Here after intravenous injection the concentration was 1/100th to 1/50th that in the lung, but after subarachnoid injection both tissues held comparable concentrations.

Summary

1. Details of the preparation of a radioactive palladium colloid are given and some of its properties are described.

2. After intravenous injection of the colloid, the rate of fall of the concentration of palladium in the blood depended partly upon the size of the dose.

3. After subarachnoid injection a small and variable concentration of colloid appeared in the blood.

4. Tissue assays after injection by either route showed that the lungs held a higher concentration than any other tissue examined except the kidney, which, after a subarachnoid dose, showed a concentration comparable with that in the lung.

The author wishes to thank Professor E. B. Verney for facilities in his laboratory. Thanks are also due to the staff of A.E.R.E., Harwell, and the Cambridge electron microscope group, and to the Medical Research Council for a grant towards the expenses of the research.

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THE CHEMOTHERAPEUTIC ACTION OF PHENANTHRIDINE COMPOUNDS

PART V

THE EFFECT OF FOUR NEW PHENANTHRIDINE DERIVATIVES UPON FLY-TRANSMITTED *T. VIVAX* INFECTIONS IN CATTLE

BY

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In Part I of this work (Brownlee, Goss, Goodwin, Woodbine, and Walls, 1950) a number of new phenanthridinium compounds were described which were at least as active as dimidium against laboratory *Trypanosoma congolense* infections in mice. Four of the best of these compounds were selected for trial in the field, in the hope that one or other of them might show advantages over dimidium in the treatment of cattle trypanosomiasis, either because of greater activity or because of absence of the toxic effects peculiar to dimidium.

The drugs were tested upon the acute stage of tsetse-fly-transmitted *T. vivax* infections in Zebu cattle kept on the plateau of N. Nigeria at Vom. Skilled assistance was limited and our object, therefore, was to obtain the maximum amount of accurate information from a group of animals small enough for us to manage by ourselves. All the observations recorded in this paper, except for the temperatures, were made personally by the authors.

MATERIALS AND METHODS

Phenanthridinium compounds

The four drugs selected were prepared by Dr. L. P. Walls and Dr. T. Dewing. They were as follows:

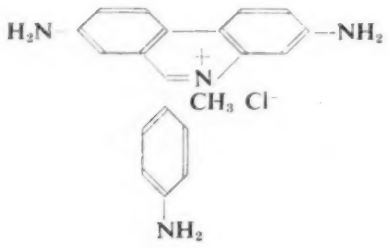
150C47—2: 7-diamino-9-*p*-aminophenyl-10-methylphenanthridinium chloride (1.5 times as active as dimidium in mice).

676C46—2: 7-diamino-9-*p*-nitrophenyl-10-methylphenanthridinium chloride (1.3 times as active as dimidium in mice).

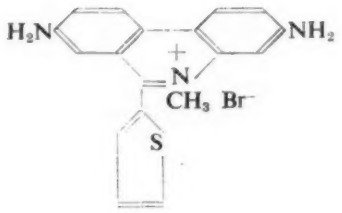
621C47—2: 7-diamino-9-α-thienyl-10-methylphenanthridinium bromide (1.3 times as active as dimidium in mice).

196C48—7-ethoxy-9-*p*-aminophenyl-10-methylphenanthridinium chloride (0.7 times as active as dimidium, and 0.5 times as toxic intravenously in mice).

* This work was done while L. G. Goodwin was a guest at the laboratories of the West African Institute for Trypanosomiasis Research from January to June, 1951.



150C47



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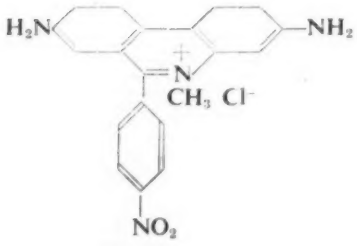
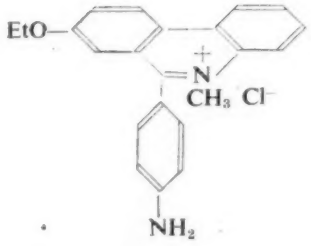


676C46



196C48

The drugs were used dissolved in water, and doses were given subcutaneously at the side of the neck. This site was chosen to avoid spoiling the hide from any irritant effects the drug might show. 150C47 was given in 5 per cent solution, 676C46 in warm 2 per cent solution, 621C47 in 1 per cent solution, and 196C48, which was only sparingly soluble, in 2 per cent suspension in water.

For comparison a group of cattle was reserved for treatment with antrycide methyl sulphate at a dose level of 5 mg./kg., injected subcutaneously in 5 per cent solution at the side of the neck.

All solutions were freshly prepared immediately before injection and aseptic precautions were taken.

Fly-transmitted infections—first experiment

Cattle.—The cattle used were the Fulani type of Zebu bulls common upon the N. Nigerian plateau. They were bought at Bokkos by the Veterinary Officer, Mr. Erik Krog, who inoculated them against rinderpest, blackquarter, and haemorrhagic septicaemia before transfer to Vom. All were approximately 18 months old, and weighed between 160 and 260 kg. They were obtained towards the end of the dry season when fodder was scarce; they were lean and in rather poor condition. During the experiment they were fed upon locally-grown hay with no supplements of any kind except for "kanwa" (natron) as a salt-lick. When the grass began to grow at the beginning of the rains, all animals, which were free from trypanosomes in the peripheral blood, were grazed out of doors during daylight; animals which showed trypanosomes were kept indoors in fly-proofed sheds and fresh-cut grass was given when available. By this means we endeavoured to treat all the animals as nearly as possible in the way they would be treated in the field. The animals remained lean until the fresh grass grew and then they put on weight rapidly.

Tsetse flies.—When the experiment was begun, supplies of laboratory-bred tsetse flies were very short and we therefore made use of wild *Glossina morsitans*. The insects were collected through the kindness of Dr. T. A. M. Nash, at Mokwa, Niger Province, on the

Jebba cattle route. Recent experience had shown that about 40 per cent of flies caught in this locality were infected with trypanosomes; both *T. vivax* and *T. congolense* were found in the area, but *T. vivax* predominated. We were fortunate in that the flies we used all produced *T. vivax* infections in cattle, although we were quite prepared to have encountered mixed infections. A total of 46 flies survived the long journey by air and road from Mokwa. They were distributed into 3 in. × 1 in. glass tubes capped with net and labelled individually. They were kept in an insectarium in which the temperature and humidity were controlled. Six weeks later, eight flies remained alive; dissection showed all of these to be free from trypanosomes.

Method of infecting cattle.—It was important to get the 24 cattle infected as uniformly as possible. Therefore, 6 animals were selected at random and each was offered to a batch of tsetse flies. The flies were allowed to feed for 5 min. while the tubes were pressed on to an area of close-clipped skin on the back. On the following 3 days different batches of 6 cattle were bitten, so that by the 4th day all 24 animals had been offered to a group of flies once. A note of the number of flies which gorged themselves at each meal offered was made; each fly fed on the average about every 2nd day, but sometimes probed even when it did not feed. On the 5th day the first batch of 6 animals were bitten again, but the groups of flies were interchanged. The permutations of flies and cattle were continued, so that by the end of 24 days all the flies would have been offered a feed on every beast. However, trypanosomes appeared in the blood of some cattle on the 14th day, and the animals which were infected were taken out of the fly-feeding routine; thus more flies became available for feeding upon the cattle which remained. Three animals already had *T. vivax* present in the blood when they arrived at the laboratory, showing that they had been recently exposed to tsetse fly. These animals were treated in exactly the same way as the rest of the herd and were bitten by the same flies. They were allocated to groups for treatment with drugs on the 14th day, when the first of the remaining cattle became infected. At the end of 30 days of fly-feeding, all the cattle had shown *T. vivax* in the peripheral blood except one (No. 24); this animal had had a rise of temperature 27 days after the beginning of the experiment, but no trypanosomes were detected in the blood. However, a heavy *T. vivax* infection was revealed by lymph-gland puncture taken on the 34th day; the animal had probably become infected at the time of the rise of temperature.

Treatment with drugs.—It was intended that the 24 animals should be divided into six groups of four, so that there would be a group to be treated with each of the new phenanthridinium compounds, a group for antrycide, and a group of controls. When a bull had shown trypanosomes in the blood for 2 days, and had a heavy infection, a dose of a drug was given subcutaneously. The group into which each animal was placed was selected by drawing numbers from a hat. When one animal had been allocated to each of the six groups, a second "round" was started, and so on until all of the animals had been used. In this way, each group contained animals which had developed their infection rapidly, and animals which had taken longer periods to become infected; also the distribution of the bulls between the groups was as impartial as possible. Two animals (Nos. 9 and 10) died before a dose of drug could be given, which meant that two of the groups, which happened to be the controls and the group treated with 196C48, contained only 3 animals each.

When a dose of drug was given, the blood became free from trypanosomes in 1–2 days; after a further few days the treated animal was allowed out of doors to graze on the plateau during the day and to sleep in a kraal at night. If an animal showed a relapse of the infection he was immediately brought into the fly-proofed shed again, to minimize the danger of mechanical transmission to other stock by biting flies. There are no tsetse flies in the neighbourhood of Vom. Animals in which the infection relapsed were given 1 mg./kg. of dimidium bromide on the other side of the neck. When the time came for one of us

(L.G.G.) to return to England, the treated cattle were all free from trypanosomes. They were grazed for a further 3 months, and after a final blood examination sold for slaughter.

Routine examinations.—Weight was estimated by measurement of the girth behind the shoulder with a special tape. The rectal temperature of each animal was taken daily in the cool of the morning. Fresh blood preparations were examined for trypanosomes almost every day, and lymph-gland punctures were taken from all of the animals at the beginning of the experiment and at approximately monthly intervals. The local reaction to the drug was measured and assessed at intervals of a week or two. Punch biopsies of the liver of selected animals were made on several occasions by a method demonstrated to one of us by Dr. Ruth Allcroft, using a trochar and cannula made by A. L. Hawkins & Co., Ltd., of New Cavendish Street, London, W.1.

Fly-transmitted infections—second experiment

A second experiment was begun in June, 1951, at the beginning of the rains. A group of 10 cattle about 12 months old was exposed to infection by *G. tachinoides* collected from Shendam and Jemaa (Plateau province). In spite of frequent exposures for many weeks, none of the animals showed trypanosomes in the peripheral blood. A further supply of the Mokwa *G. morsitans* was then obtained, and with these all but one of the cattle were successfully infected. Some of the animals had mixed infections of *T. congolense* and *T. vivax*. The drugs used in this experiment were 150C47 and antrycide methyl sulphate. Both were given at a dose level of 0.5 mg./kg. so that a direct comparison of activity could be made. Some of the animals were left for a longer period than 2 days from the first appearance of trypanosomes before the drug was given.

Syringe-transmitted T. congolense *infections.*—A small experiment was made with 4 calves about 12 months old, bred at the Veterinary Department Stock Farm at Vom. They were infected with *T. congolense*, transmitted by subcutaneous injection of blood from a heavily infected rat. This strain of *T. congolense* was isolated from a dog in Kaduna in November, 1951, and the 6th rat passage was transferred to the calves. All showed trypanosomes in the blood by the 9th day, and the temperatures were raised. They were treated after 4 days of parasitaemia with 1 mg./kg. of 150C47, 1 mg./kg. of dimidium, 2 mg./kg. of 196C48, and 5 mg. kg. of antrycide methyl sulphate respectively. Blood and lymph-gland examinations and liver biopsies were made as in the first experiment.

Results

The primary infection with trypanosomes was marked by a rise in temperature to 102–105° F. This rise did not occur in two of the three cattle which were already harbouring the infection (Nos. 4 and 5).

The three control animals showed *T. vivax* infections which developed in a manner typical of the disease. One (No. 11) died in the acute phase and one (No. 17) died after 3 months of fluctuating temperature and parasitaemia. The third (No. 15), which was already infected with *T. vivax* on arrival, showed a peak of temperature and trypanosomes in the blood from time to time for 6 months and then passed into a condition of " cryptic trypanosomiasis " (Fiennes, 1950). This animal was eventually killed 12 months after the beginning of the experiment. A succession of small peaks of temperature recurred at intervals of 3 to 4 weeks, and these were associated with the appearance of trypanosomes in larger numbers in the peripheral blood. They suggest some form of cyclical process in the host-parasite relationship.

The results of treatment with drugs are summarized in Table I. The treated animals showed rapid and sometimes profound falls of temperature, which occurred

TABLE I

THE EFFECT OF TREATMENT UPON INFECTED ANIMALS

Group No.	Treatment	Bull No.	Weight (kg.)		Notes
			When dosed	In June, 1951	
I	Controls	11	203	—	Died 8 days after appearance of trypanosomes
		15	203	192	Infected on arrival at Vom. Showed trypanosomes for 6 months from time to time; finally killed 12 months after beginning of experiment
		17	192	—	Died 12 weeks after appearance of trypanosomes
II	150C47 1 mg./kg.	1	181	214	Free from trypanosomes until end of the experiment, 7 months after infection
		7	186	203	
		21	220	316	
		22	230	250	
III	196C48 2 mg./kg.	6	214	—	Died 3 days after dose. Severe local reaction
		8	160	160	Severe local reaction. Relapsed 44 days after dose
		13	210	214	Severe local reaction. Relapsed 15 days after dose
IV	676C46 1 mg./kg.	2	263	293	Relapsed 35 days after dose
		16	220	238	Free from trypanosomes until the end of experiment
		18	190	214	
		19	210.	—	Died 17 days after dose
V	621C47 1 mg./kg.	4	214	232	Infected on arrival. Freed from trypanosomes until end of experiment
		24	190	203	Freed from trypanosomes until the end of experiment
		26	220	263	
		29	200	250	
VI	Antrycide methyl sulphate 5 mg./kg.	3	170	—	Died 12 days after dose
		5	250	—	Died 19 days after dose
		23	238	270	Freed from trypanosomes until the end of experiment
		25	200	238	

even in Nos. 4 and 5, which had longer-standing infections and normal temperatures at the time of injection of the drug.

Phenanthridinium compounds.—All the cattle which received 1 mg. per kg. of 150C47 or of 621C47 were apparently cured; they never again showed a trypanosome in the blood or lymph gland juice, and their temperatures remained normal for the rest of the experiment. In addition, the animals put on a considerable amount of weight.

Of the four animals which were given 1 mg. per kg. of compound 676C46, one relapsed and one died. This drug was therefore less effective and possibly more toxic to cattle than 150C47 or 621C47.

All three animals treated with 196C48 relapsed, and two were subsequently cured with 1 mg. per kg. of dimidium bromide. Compound 196C48 was effective in mice and was less toxic by subcutaneous injection than dimidium; we thought

that it might have advantages when given in 2 mg. doses, but this did not prove to be true. Moreover, the substance produced enormous oedematous swellings at the site of injection. The lower subcutaneous toxicity to mice is probably associated with a slower rate of absorption from the site of injection which in turn may be responsible for the more severe local reaction. Although there was considerable variation between individual animals it appeared that 150C47, 676C46, 621C47, and dimidium were about equally irritant at the site of injection. In most of the cattle the drugs produced a hard lump which sometimes gave the animals sufficient discomfort to make them rub their necks on a post or fence. When this occurred the hair was shed and the skin damaged. In the majority of animals all that remained was a patch of bare thickened skin. In three animals, however (Nos. 1, 13, and 22), an area of necrosis was produced which took several weeks to heal. No dressings were applied; the lesions were left uncovered and had all healed at the end of four months. In contrast to these, there were other animals, treated in exactly the same manner, which showed only very slight local reactions. The degree of reaction could not be correlated with the age or colour of the beast, or with any other property we could observe.

Examination of the tissues of the two animals which died showed that No. 6, which succumbed to treatment with 196C48 (2 mg. per kg.), had necrosis of the liver parenchyma around the central veins, and some scattered patches of fatty infiltration. There were also basophilic concretions in the kidney collecting tubules. It is likely that this animal was killed by the drug. No. 19, which died after 1 mg. per kg. of 676C46, had areas of normal liver parenchyma, but the sinusoids and veins were crammed with inflammatory cells. Some areas of the liver were putty-like in consistency, and there were numerous *Schistosoma bovis* worms in the portal venous system. The lungs were congested and the apical lobes consolidated. It seems unlikely that the death of this animal was caused by the drug; the bull certainly had a bacterial infection of some kind.

No treated animal in any group showed evidence of jaundice or of " photosensitization," although it was in the Veterinary Laboratories at Vom that some of the very first occurrences of " photosensitization " after dimidium were observed. The light intensity during the first 3 months of the present experiment was very high; a record obtained from the Meteorological Station at Jos airport, 17 miles from Vom, showed that there was an average of 10 hours' sunshine every day.

Antrycide.—Of the 4 cattle treated with 5 mg. per kg. of antrycide methyl sulphate, two were apparently cured. The other two bulls died 12 and 19 days respectively after the dose. Death followed an illness of a type we had previously observed in cattle which died after injections of antrycide prophylactic mixture, which contains a 5 mg. per kg. dose of the methyl sulphate in addition to antrycide chloride. The chief clinical sign was oedema, which appeared after 6–10 days in the tissues of the anus and the genitalia, later in the lower abdominal wall, and spread finally to the tissues of both hind limbs and the rear half of the body. The urine contained protein. Both animals showed gross oedema of the perirenal tissues and the pelvic wall *post mortem.* There was excessive free fluid in the abdominal, thoracic, and pericardial cavities. Bull No. 5 had also bled profusely into the pelvic tissues and into the abomasum, which contained about a litre of blood clot. Acute haemorrhagic gastritis following toxic doses of antrycide methyl sulphate has also been

reported by Wilson (1949) and by Garner (1950). Burdin and Plowright (1952) have also recorded kidney damage in antrycide poisoning.

Sections of the kidneys of the animals which died showed degeneration of the tubules; many of the collecting tubules were dilated and obstructed with casts (Fig. 1). The glomeruli were not severely affected. The liver and endocrine organs were apparently normal, but the muscle fibres of the heart were filled with fine droplets of fat; both animals harboured *Sarcocystis* in the heart muscle.

The local reaction to injection of antrycide methyl sulphate was less severe than the reactions produced by the phenanthridinium compounds. This was an unexpected finding, because preliminary tests in English cattle showed no significant differences between the irritant properties of dimidium and antrycide (Goodwin and Walls, 1950).

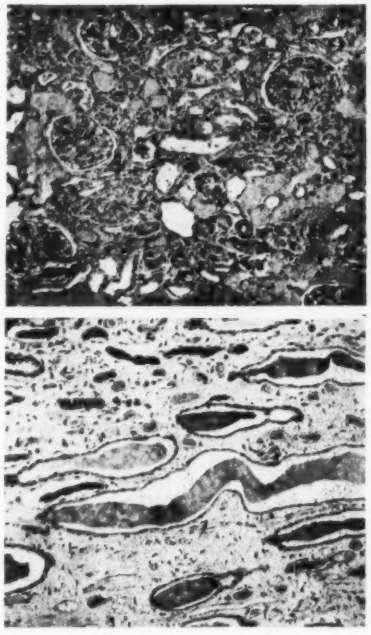


FIG. 1.—Kidney of bull No. 3, which died with severe oedema and proteinuria 12 days after a dose of 5 mg. per kg. of antrycide methyl sulphate. The cortex shows degenerating tubules; the medulla shows the collecting tubules to be dilated and to contain granular casts. Haematoxylin and eosin (× 75).

Unexplained deaths.—Two of the animals died before any drug was given; one of six cattle from the same herd as the test animals, which were neither exposed to tsetse nor treated with drugs, also died. The fact that some of the cattle died before drugs were given makes it necessary to regard with reservation the deaths in the groups which were treated. Fulani cattle which have spent all of their lives out of doors do not take kindly to being shut inside a fly-proofed cowshed. Some pine away and die. However, bulls Nos. 3, 5, and 6 showed definite pathological changes which could be attributed to the action of a poison, so that we must conclude that the treatment made some contribution towards the death of these animals. Fatty droplets in the heart muscle or basophilic concretions in the kidney medulla could not be regarded as reliable evidence of poisoning by drugs, because these lesions were found in untreated control animals.

The results of liver biopsy.—By the method of liver biopsy it was possible to get an idea of the condition of the livers of cattle during the dry season (the time of the lowest level of nutrition) and also to note any effects of the drugs upon the organ when animals have been treated. The specimens obtained showed no gross abnormalities and no changes which could be attributed to the action of any of the drugs given. In Part VI of this work we have studied more fully the toxic effects upon the liver of larger doses of 150C47 (Goodwin and Chandler, 1952).

The second experiment (Table II) showed clearly that at a dose of 0.5 mg. per kg. neither phenanthridinium compound 150C47 nor antrycide methyl sulphate cured *T. vivax* infections. 150C47 was at least as effective as an equal amount of

TABLE II

THE EFFECT OF SMALL DOSES OF 150c47 AND ANTRYCIDE METHYL SULPHATE UPON FLY-TRANSMITTED TRYPANOSOME INFECTIONS

Treatment	Bull No.	No. of days infected before dose	Notes
Controls	54	—	Died 17 days after first appearance of *T. vivax*
	56	—	Alive but infected with *T. vivax* at the end of 113 days
150C47 0.5 mg./kg.	57	1	Original infection of *T. vivax* relapsed 16 days after dose
	58	37	Infected with *T. vivax* and *T. congolense*; died from causes other than trypanosomiasis after 35 days
	59	17	Infected with *T. vivax*; relapsed 19 days after dose
	63	20	Infected with *T. vivax* and *T. congolense*; relapsed with *T. vivax* 22 days after dose
Antrycide methyl sulphate 0.5 mg./kg.	55	1	Infected with *T. vivax*; relapsed 7 days after dose
	60	37	Infected with *T. vivax*; relapsed 22 days after dose
	64	1	Infected with *T. vivax*; relapsed 6 days after dose

antrycide when judged by the period that the animals remained free from trypanosomes in the peripheral blood before they relapsed. The length of time that the infection had been present before treatment did not seem to influence the effect of either drug in the small number of animals studied. A donkey naturally infected with *T. congolense* was, however, cured by a dose of 0.5 mg. per kg. of 150C47; there was no reaction at the site of injection.

The results of the experiment upon syringe-transmitted *T. congolense* infection (Table III) confirmed the conclusions drawn from the experiments upon fly-transmitted *T. vivax*. The animal treated with 196C48 relapsed after 17 days and was subsequently cured with 2 mg./kg. of 150C47. Although the local reaction to the first drug was severe, the second produced only a very small swelling. The calves

TABLE III

THE EFFECT OF PHENANTHRIDINIUM COMPOUNDS AND ANTRYCIDE METHYL SULPHATE UPON SYRINGE-TRANSMITTED *T. congolense* INFECTIONS

Treatment	Calf No.	Weight (kg.)			Notes
		Feb. 7	May 8	June 19	
150C47 1 mg./kg.	I	127	120	127	Free from trypanosomes until the end of experiment, 8 months after infection
Dimidium bromide 1 mg./kg.	III	120	99	123	Free from trypanosomes until the end of experiment, 8 months after infection
196C48 2 mg./kg.	V	99	116	120	Severe local reaction. Relapsed 17 days after dose. Subsequently cured with 2 mg./kg. of 150C47
Antrycide methyl sulphate, 5 mg./kg.	VI	127	124	155	Free from trypanosomes until the end of experiment

treated with 1 mg. per kg. of 150C47, 1 mg. per kg. of dimidium bromide, and 5 mg. per kg. of antrycide methyl sulphate respectively, remained free from trypanosomes in the peripheral blood and lymph gland juice until they were sold 8 months afterwards. The local reaction to antrycide in calf VI was more severe than those observed in the larger animals and took about 6 weeks to resolve. The weights of all the animals decreased during the dry season, but increased again when the grazing improved at the beginning of the rains. Liver biopsy specimens showed no significant pathological changes during the first 5 months of the experiment.

Discussion

The experiments described above showed that two of the new phenanthridinium compounds were effective in the treatment of fly-transmitted *T. vivax* infections in cattle. The other two compounds, although promising in the laboratory, were of little use under field conditions. The animals which relapsed after treatment with 676C46 and 196C48 were subsequently cured by a dose of 1 mg. per kg. of dimidium, which showed that the strain of *T. vivax* used was normally sensitive to this drug. Our main reason for using only a small number of cattle was so that we should be able to make a careful preliminary study of the effects of the drugs upon a uniform fly-transmitted infection. Such a study is of more value than a less carefully controlled experiment upon a larger herd of cattle.

The conclusions to be drawn from the results as they stand is that 150C47 and 621C47 are active drugs with potencies of the same order as that of dimidium. In the dosage used, they caused no serious side-reactions, either immediate or delayed, in cattle which were in poor condition. The effects of larger, toxic doses of 150C47 are described in Part VI of this work (Goodwin and Chandler, 1952). A further study of 621C47 is at present in progress and it remains to be seen whether or not this compound will show any advantages over dimidium. The finding of Burdin and Plowright (1952) that it has considerably lower toxicity to the liver as judged by serum bilirubin, serum alkaline phosphatase, and liver biopsy specimens suggests that it may find a place in the treatment of cattle trypanosomiasis.

Antrycide methyl sulphate at a dose of 5 mg./kg. was effective against the strain of *T. vivax* used, but killed two of the four animals treated because of a toxic effect upon the kidney. When animals are in poor general condition they do not tolerate large amounts of potent medicines. It appears that the margin of safety between the therapeutic and toxic doses of antrycide is not as great as was at first thought, especially when the animals are in poor health. This will be particularly troublesome in areas where strains of trypanosome are encountered which are more than usually resistant to the drug.

Summary

1. Four new phenanthridinium compounds have been tested against tsetse-fly-transmitted *Trypanosoma vivax* infections in Nigerian cattle. Antrycide methyl sulphate was used as a standard of comparison.

2. Two compounds, 150C47 (2: 7-diamino-9-*p*-aminophenyl-10-methylphenanthridinium chloride) and 621C47 (2: 7-diamino-9-α-thienyl-10-methylphenanthri-

dinium bromide) cured infections of *T. vivax* at a dose level of 1 mg. per kg.; doses of 0.5 mg. per kg. were not curative.

3. The other two compounds, 196C48 (7-ethoxy-9-*p*-aminophenyl-10-methyl-phenanthridinium chloride) and 676C46 (2: 7-diamino-9-*p*-nitrophenyl-10-methyl-phenanthridinium chloride), were less active and possibly more toxic. No cases of photosensitization were observed after any of these four compounds.

4. Antrycide methyl sulphate at a dose of 5 mg. per kg. cured two of four cattle. The other two animals died as a result of damage to the kidney tubules by the drug. A dose of 0.5 mg. per kg. of antrycide was not sufficient to cure *T. vivax* infections.

5. It was concluded that 150C47 and 621C47 deserved further study.

We wish to express our thanks to Brigadier J. S. K. Boyd and to Col. H. W. Mulligan for their interest and encouragement, and to Mr. W. G. Beaton, Mr. R. S. Marshall, and Mr. E. Krog, of the Nigerian Veterinary Service, for many acts of kindness in arranging a supply of animals, for some laboratory equipment and facilities, and for the personal accommodation of L.G.G. We also wish to thank Dr. T. A. M. Nash, who made arrangements for the collection and transport of wild *G. morsitans* from Mokwa, and Dr. L. P. Walls and Dr. T. Dewing, who prepared the phenanthridinium compounds.

We are very grateful for the help of Mr. P. E. Nesbitt, who prepared sections of the liver biopsy specimens, and of Mr. H. Richards, who dealt with a large amount of post-mortem material.

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THE CHEMOTHERAPEUTIC ACTION OF PHENANTHRIDINE COMPOUNDS

PART VI

THE PROPHYLACTIC AND TOXIC ACTIONS OF 2:7-DIAMINO-9-*P*-AMINOPHENYL-10-METHYLPHENANTHRIDINIUM CHLORIDE (150C47) AND IODIDE

BY

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In Part V of this work (Goodwin and Unsworth, 1952) it was shown that two new phenanthridinium compounds were active against trypanosome infections in Fulani cattle at a dose of 1 mg./kg. Although the earlier phenanthridinium compounds, dimidium and phenidium, have proved disappointing as prophylactics, it was thought worth while to make a trial with one of the new drugs. No toxic effects had been observed with the 1 mg./kg. dose and we thought that larger doses would be tolerated; also we wished to study the toxic action of the drug when the dose was raised above the limit of tolerance. In addition, we had at our disposal an oily suspension of the sparingly soluble iodide corresponding to 150C47 (2:7-diamino-9-*p*-aminophenyl-10-methylphenanthridinium iodide), which, by analogy with antrycide chloride, we thought might form a depot and exert a trypanocidal action for a prolonged period. Previous work by Unsworth had shown that at Lumu on the borders of the Ribako forestry reserve (Zaria province of N. Nigeria), where there was very heavy infestation with *Glossina morsitans*, untreated cattle became infected with *Trypanosoma vivax* and *T. congolense* in 3–6 weeks and died in 1–3 months. Antrycide prophylactic mixture injected at intervals of 90 days was powerless to prevent infection of the majority of cattle in this area, although it prolonged the survival time of the animals (Unsworth, 1951). The grazing at Lumu was very good; there was an extensive river plain (fadama) covered with good grass along the borders of a tributary of the Kaduna river.

The experiment described below showed that the prophylactic activity of 150C47 was poor, and that at doses of 2 mg./kg. and over the drug had toxic effects.

MATERIALS AND METHODS

Drugs.—150C47—2:7-diamino-9-*p*-aminophenyl-10-methylphenanthridinium chloride was prepared in fresh 5 per cent (w/v) solution and injected subcutaneously on the side of the neck in doses of 2 or 3 mg./kg.

* This work was done while L. G. Goodwin was working as a guest at the West African Institute for Trypanosomiasis Research.

65C51—The corresponding iodide in 15 per cent (w/v) suspension in arachis oil was injected similarly in doses of 3, 5, or 10 mg./kg.

These drugs were prepared by Dr. L. P. Walls.

Cattle.—Sixteen Fulani bulls about 12 months old and weighing 102–181 kg. were purchased for us at Bokkos on the plateau by Mr. Erik Krog. They were inoculated against blackquarter and haemorrhagic septicaemia. They then travelled on the hoof to Vom, where blood and lymph-gland examinations were made and injections of the drugs were given. Examination of the blood revealed that two animals were already infected with *T. vivax*. Three bulls were selected at random from among the uninfected animals to serve as untreated controls; the rest were distributed into groups for treatment by drawing numbers from a hat. The cattle were rested overnight after the dose and during the next 6 days walked 160 miles through the bush to Lumu. Riverine tsetse (*Glossina palpalis* and *G. tachinoides*) were encountered soon after leaving the plateau and attacked the cattle at the shady crossings of the watercourses. *G. morsitans* appeared about 40 miles from Lumu. None of the cattle died on the journey; all arrived in fairly good condition and were sent to graze daily in the Ribako forestry reserve.

Examinations.—The cattle were left at Lumu in the charge of herdsmen who had had several years' experience of similar experiments. Twice a week, at least one of the authors travelled to Lumu to make blood examinations (fresh preparations and thick and thin smears) and to assess the condition of the animals. Lymph-gland punctures, liver biopsies, and weight assessment were made on several occasions, and the local reactions at the sites of injection were carefully noted and recorded. If an animal fell sick, one of us (R.L.C.) went as quickly as possible to make an examination, and if necessary to slaughter and post-mortem the animal. Samples of the organs were fixed in formol-saline. Frozen and paraffin sections were prepared and stained in England.

Survey of plants.—When it was seen that the treated cattle were dying, we made a survey of the vegetation of the area to see if there were any known poisonous plants which might have contributed to the toxic effects of the drug. We attempted to identify as many as possible of the grasses, herbs, shrubs, and trees in the area which were available to the cattle. The plants which were in flower were collected and identified in the fresh state by reference to the *Flora of West Tropical Africa* (Hutchinson and Dalziel, 1928). The plants which were not in flower were more difficult to identify, so we made a collection in the company of a party of African herdsmen who knew the district. The Hausa name of each plant was recorded, together with any information as to its reputed virtues or poisonous properties. With this record, and a careful examination of the plant, it was possible to identify all but a very few with reasonable certainty by reference to the list of vernacular names and the information in the appendix to the *Flora* (Dalziel, 1948). The habitats of the plants collected fell into three distinct types: (*a*) the grassy river-plain, (*b*) the tree-savannah (secondary forest) which adjoins the plain, and (*c*) savannah clearings which had been farmed by the local population. Plants in all of these areas were available to the animals every day during the time of grazing. A list of the species identified is given in Table III. For the whole period of the experiment there was a good supply of green grasses on the river plain.

RESULTS

Local reaction.—The local reaction at the site of injection of the drug was similar to that already described by Goodwin and Unsworth (1952). There was no difference between the effect of the chloride and iodide; large doses of drug produced more severe reactions than smaller ones.

Prophylactic action of 2: 7-*diamino*-9-p-*aminophenyl*-10-*methylphenanthridinium salts*

Table I shows that the untreated control animals showed trypanosomes in the blood after 19, 33, and 47 days respectively. Two of them died. Of the 13 treated animals, 10 died from the toxic effects of the drug, and two of the three survivors showed trypanosomes in the blood 72 days and 73 days respectively after exposure

TABLE I

PROPHYLACTIC ACTION OF 150C47 (SOLUBLE CHLORIDE) AND 65C51 (INSOLUBLE IODIDE)

Drug	Bull No.	No. of days free from trypanosomes	Other notes
Controls ..	32	19	Killed *in extremis* after 73 days (*T. congolense* and *T. vivax*)
	37	47	Survived 78 days (*T. congolense*)
	44	33	Killed after 66 days, with heavy trypanosome infection (*T. congo.* and *T. vivax*) and abscess of abdominal wall
10 mg./kg. iodide	46	Until death	Killed *in extremis* 32 days after dose
5 mg./kg. iodide	31	Until death	Died 39 days after dose
	36	Until death	Killed *in extremis* 37 days after dose
	45	Until death	Died 32 days after dose
3 mg./kg. iodide	34	Until death	Killed *in extremis* 39 days after dose
	39	Until death	Died 34 days after dose
	43	73	Survived, uninfected
3 mg./kg. chloride	35	72	Survived, but *T. vivax* present in blood
	40	Until death	Killed *in extremis* 32 days after dose
	42	Until death	Killed *in extremis* 39 days after dose
2 mg./kg. chloride	33	73	*T. vivax* infection; the animal was weak and was killed
	38	Until death	Died 17 days after dose
	41	Until death	Killed *in extremis* 27 days after dose

to the tsetse fly. The remaining animal (No. 43), which had received 3 mg./kg. of the sparingly soluble iodide, remained free from infection for the whole of the 73 days spent in the fly-infested area. The prophylactic value of the compound was not impressive.

Toxic effects

A treated animal gave very little previous indication that he was shortly going to die. One day he would lag a little behind the others while grazing; the next morning he would be unable to rise and in a further 1–3 days he would die or be so ill as to require slaughtering. The animal would continue to eat until too weak to do so. On no occasion was jaundice, or dermatitis suggestive of photosensitization, observed, although we examined the animals very carefully with these possibilities in mind. Examination of the urine of bulls 39 and 41, collected shortly before death, revealed no protein, reducing substances, or bile pigments. We had no facilities for studying the amino-acids present in the urine. A record of the sunshine

and rainfall during the period of the experiment was obtained from the Meteorological station at Kaduna airport, 30 miles from Lumu. This showed that there was no lack of sunshine; the light intensity during this period of the year in N. Nigeria is very high. The post-mortem appearances and significant histological findings in the organs of cattle which died from the toxic effects of the drug are summarized in Table II. The animals which died showed lesions in the liver, the kidney, and

TABLE II

SUMMARY OF PATHOLOGICAL FINDINGS IN ANIMALS WHICH DIED

Drug	Bull No.	Liver						Kidney				Heart		Worms		
		1	2	3	4	5	6	1	2	3	4	1	2	1	2	
Controls	32		+										+			
	37															Survived
	44		+													
10 mg./kg. iodide ..	46						+	+	+	+	+	+	+	+	+	
5 mg./kg. iodide ..	31	+	+	+	+	+		+				+		+	+	
	36	+	+	+	+			+	+	+	+	+	+	+	+	
	45	+	+	+	+	+	+	+	+	+				+	+	
3 mg./kg. iodide ..	34			+				+	+	+			+		+	
	39	+	+	+		+		+					+	+	+	
	43			+	+	+										Survived
3 mg./kg. chloride ..	35															Survived
	40			+	+			+	+	+		+		+	+	
	42	+		+				+				+	+		+	
2 mg./kg. chloride ..	33		+									+	+			
	38		+			+							+		+	
	41	+	+						+			+		+	+	

KEY.

Liver
1—Thick, dark bile.
2—Deposit of bile pigment in liver section.
3—Slight fatty infiltration.
4—Gross fatty infiltration.
5—Central necrosis.
6—Pink-staining lumps.

Kidney
1—Vascular congestion.
2—Protein deposit in Bowman's capsule.
3—Fat in some tubule cells.
4—Casts.

Heart
1—Fat droplets in muscle fibres.
2—Sarcocystis.

Worms
1—Liver flukes.
2—Stomach worms.

the heart. No significant abnormalities were observed in the gastro-intestinal tract, the lungs, or the endocrine glands. Most of the animals carried very heavy worm infestations.

Liver.—The lesion in the liver was a fatty infiltration, which sometimes affected all parenchymal cells (Fig. 1). In some cases there was centrilobular necrosis, and collapse of the reticulin framework in the central areas (Fig. 2). Some animals showed only a slight fat deposit, limited to the cells near the portal tracts) Several livers showed heavy bile pigmentation, and some (Nos. 46, 45, and 38. showed the presence of lumps of hyaline material which stained faintly pink with Sudan III.

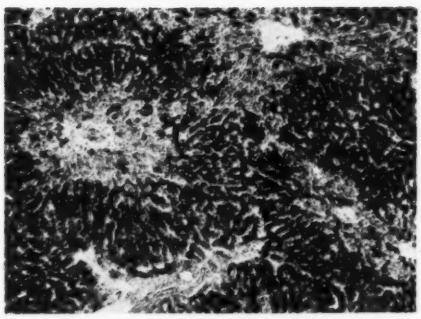

FIG. 1.—Liver of calf No. 31, showing fatty infiltration. Sudan III, haematoxylin, and aniline blue (× 38).

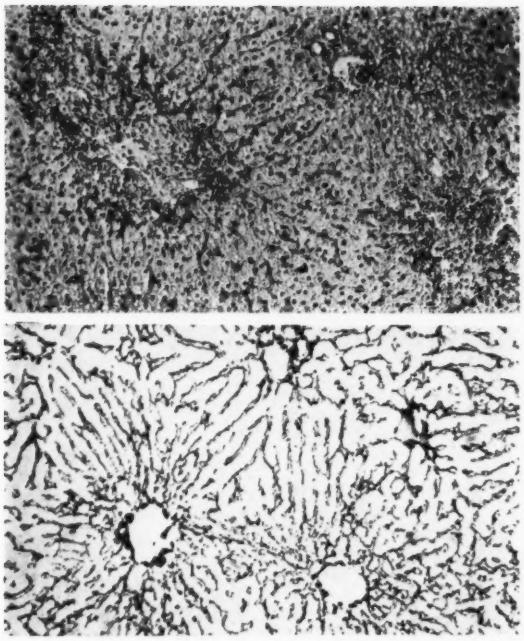

FIG. 2.—Liver of calf No. 31, showing fatty infiltration, centrilobular necrosis, and collapse of reticulum. Upper figure—haematoxylin and eosin; lower figure—modified Foot (× 75).

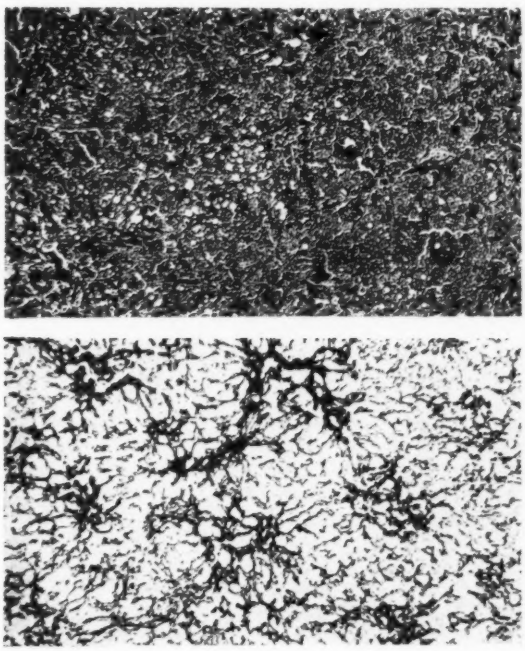

FIG. 3.—Liver of calf No. 43. Biopsy specimen taken 7 weeks after a dose of 3 mg./kg. of 65C51. The liver architecture is destroyed and the reticulum condensed; there are numerous fatty cysts. Upper figure—haematoxylin and eosin; lower figure—modified Foot (× 75).

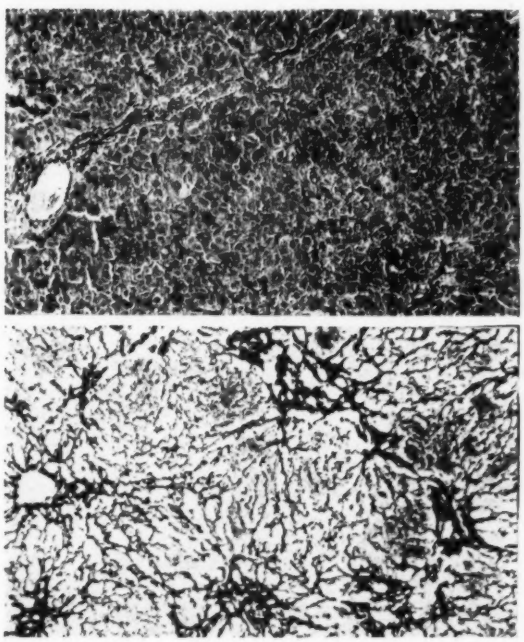

FIG. 4.—Liver of calf No. 43. Biopsy specimen taken 11 weeks after a dose of 3 mg./kg. of 65C51. The liver parenchyma and bile ducts have regenerated, but the architecture is abnormal. Upper figure—haematoxylin and eosin; lower figure—modified Foot (× 75).

The effect of the drug upon the liver in the animal which recovered (No. 43) was well shown by serial liver biopsies. Seven weeks after the dose the liver was heavily infiltrated with fat, and there was formation of fatty cysts similar to those described in choline-deficient rats by Hartroft (1950, 1951). The architecture of the liver had been severely affected (Fig. 3). In a further four weeks there was some regeneration of liver tissue (Fig. 4) although the reticulin stain showed that the architecture was abnormal. The animal may well have been developing a diffuse fibrosis (Himsworth, 1947).

Kidney.—In the kidneys which were affected, there was sometimes a slight protein deposit in some of the capsular spaces, and occasional nephrons showed fat droplets in the tubule cells. This may have been secondary to the fatty infiltration of the liver (Hartroft, 1951). Only in No. 46 and No. 36 was there any evidence of cast-formation. On the whole, the lesions in the kidney appeared to be slight, except in the animals which were given very large doses of drug.

Heart.—Several animals showed a generalized infiltration of the muscle fibres of the heart with minute droplets of fat. Similar fatty infiltration was observed after a lethal dose of phenamidine and in animals which died from other causes (Goodwin and Unsworth, 1952). It cannot therefore be stated with certainty that the drug was responsible for the condition of the heart muscle. Many of the animals showed extensive invasion of the muscle with *Sarcocystis*.

Worm infestation.—Most of the animals which died showed very heavy infestation with worms. Paramphistomes were present in the rumen and trichostrongyles in the abomasum, sometimes in huge numbers, and most livers contained flukes (*Dicrocoelium*). It is interesting that only a few worms were found in the untreated control animals.

Plants.—Table III is a list of the plants found at Lumu which were available to the cattle as food. Of course, this list is not a complete one; it may well be that we have missed highly poisonous species with a limited local distribution, plants

TABLE III

I. HERBACEOUS PLANTS

Acanthaceae: *Nelsonia campestris.*
Ampelidaceae: *Cissus populnea.*
Asclepiadaceae: *Glossonema nubicum, Raphionacme Brownii, Asclepias lineolata.*
Cochleospermaceae: *Cochleospermum tinctorium.*
Commelinaceae: *Cyanotis angusta, C. bulbifera, C. lanata.*
Compositae: *Aspilia latifolia, Chrysanthemum procumbens, Echinops longifolius, Gynura cernua.*
Convolvulaceae: *Ipomoea reptans.*
Cyperaceae: *Carex* sp., *Cyperus* sp., *Fimbristylis dichotoma, Kyllinga erecta.*
Euphorbiaceae: *Euphorbia hirta.*
Gramineae: *Acroceras* sp., *Andropogon* sp., *Digitaria* sp., *Echinochloa* sp., *Eragrostis* sp., *Heteropogon* sp., *Hyparrhenia rufa, Imperata* sp., *Jardinea* sp., *Lasiurus* sp., *Paspalidium geminatum, Paspalum scrobiculatum, Pennisetum* sp., *Sporobulus minutiflorus, S. spicatum, Urelytrum* sp.
Iridaceae: *Gladiolus unguiculatus, Romulea* sp.
Juncaceae: *Juncus capitatus.*
Liliaceae: *Anthericum* sp., *Urginea altissima.*
Orchidaceae: *Eulophia adenoglossa, E. cullulata, E. lutea.*
Papilionaceae: *Crotalaria Perrottetii, Tephrosia elegans.*
Rubiaceae: *Mitracarpum verticillatum.*

Solanaceae: *Schwenkia americana, Physalis angulata.*
Sterculiaceae: *Waltheria americana.*
Thymelaceae: *Arthrosolen chrysanthus.*
Verbenaceae: *Stachytarpheta angustifolia.*
Zingiberaceae: *Costus spectabilis, Kaempferia* sp.

II. TREES AND SHRUBS

Anonaceae: *Anona senegalensis.*
Araliaceae: *Cussonia nigerica.*
Caesalpinaceae: *Bauhinia Thonningii, Daniella Oliveri, Isoberlinia doka.*
Combretaceae: *Combretum sokodense, Terminalia avicennioides, T. glaucescens.*
Euphorbiaceae: *Uapaca guineense.*
Loganiaceae: *Strychnos spinosa.*
Mimosaceae: *Entada sudanica, Parkia filicoidea.*
Moraceae: *Ficus glumosa, F. kawuri.*
Myrtaceae: *Syzygium guineense.*
Ochnaceae: *Lophira alata.*
Olacaceae: *Ximenia americana.*
Palmeae: *Borassus flabelliformis.*
Papilionaceae: *Afrormosia laxiflora, Tephrosia Vogelii.*
Proteaceae: *Protea Elliottii.*
Rubiaceae: *Crossopteryx febrifuga, Gardenia erubescens.*
Sapotaceae: *Butyrospermum Parkii.*
Sterculiaceae: *Sterculia tomentosa.*
Verbenaceae: *Vitex Cienkowskii.*

which were small, or plants which had completed their season of growth and were dry at the time of collection.

A few of the species collected call for comment. The genus *Crotalaria* is well known as a cause of poisoning to stock in S. Africa (Steyn, 1934; Steyn and van der Walt, 1945; Steyn, 1949), and is known to cause liver lesions. *Urginea altissima* is also known to be poisonous (Steyn, 1934, 1945, 1949); this was growing in abundance on the river plain. One of the herdsmen regarded *Gladiolus unguiculatus* as an undesirable food plant for cattle; this too was common on the plain. No published records of the properties of many of the species recorded in Table III are available, but in a personal communication Prof. D. G. Steyn states that, in addition to the plants mentioned above, the following genera contain known poisonous plants in South Africa and elsewhere: *Cissus, Asclepias, Ipomoea, Euphorbia, Tephrosia, Arthrosolen, Terminalia, Strychnos.* Specific inquiry for *Tribulus terrestris* drew the unanimous reply from the herdsmen that this plant was not found in the Lumu area.

DISCUSSION

The phenanthridinium compound dimidium is known to have toxic effects upon the livers of cattle when given in doses only a little in excess of the therapeutic range. A dermatitis resembling that of photosensitization, and appearing several weeks after the dose of drug, is seen in some parts of Africa (Bell, 1945; Randall and Beveridge, 1946, 1947; Lederman, 1950). The occurrence of photosensitization also suggests toxicity to the liver, because " Geeldikkop," a disease of sheep in South Africa caused by eating poisonous plants such as *Tribulus terrestris*, is characterized by liver insufficiency which allows a photosensitizing derivative of chlorophyll to accumulate in the blood. The whole question of photosensitization has been fully discussed by Clare (1952).

Study of the delayed toxic effects of phenanthridinium compounds is made difficult by the fact that as yet they have not been produced in small laboratory animals. Photosensitization, jaundice, and severe damage to the liver have not been demonstrated in mice, rats, guinea-pigs, or rabbits, even after large and repeated doses of dimidium. It appears that it is essential to perform all investigations of toxicity upon cattle. A careful study in the Belgian Congo by Lederman (1950) led to the conclusion that the drug itself was responsible for the toxic action, but that the presence of other factors might influence the incidence of fatalities.

In the present experiment, although photosensitization did not occur, there is no doubt that 150C47 (which is closely related to dimidium) caused damage to the liver. Burdin and Plowright (1952) recently examined the action of phenanthridinium compounds on African cattle at Kabete, Kenya, and showed that the 10-methyl derivatives dimidium, 150C47, and, to a lesser extent, 621C47 all caused a loss of body weight and a rise in serum bilirubin and alkaline phosphatase. The structure as well as the function of the liver was affected; serial liver biopsies showed infiltration with fat. The present study agrees with the East African results, and it is clear that 150C47 has no apparent advantages over dimidium for the treatment of cattle trypanosomiasis. It has slightly greater activity, but its toxicity is of the same order and of the same type. However, it may be of use to consider the factors which may contribute to the toxicity of the drug, and which may account for the apparently contradictory reports upon the dangers of treating cattle with dimidium which have come from different parts of Africa.

The contributory causes of toxicity which have been suggested may be listed as follows: (1) Variation in the quality of batches of the drug. (2) A poor state of nutrition of the cattle. (3) The presence of toxic plants in the diet. (4) The presence of photodynamic substances in the diet. (5) The "lighting up" of an unspecified hepatotropic virus. (6) The presence of worm infestation or *Babesia* infection.

Variation in batches of the drug are not very likely to occur because the drug is a crystalline chemical compound of fixed constitution. Brownlee, Goodwin, and Walls (1947) re-examined a selection of the original samples of drug sent to Africa and could detect no differences in their toxicity or trypanodical activity in laboratory animals. Also, in the study of a large series of phenanthridinium derivatives by Brownlee, Goss, Goodwin, Woodbine, and Walls (1950) it was shown that the toxicities of all the compounds were of the same order. It is difficult to envisage an impurity which could be present and which would have a greater toxicity than dimidium itself. It is true that from some commercial batches of dimidium a precipitate forms when a sterile solution is allowed to stand for some weeks (personal communication and sample from Mr. S. G. Laws, Entebbe). Examination by Dr. L. P. Walls of the very small amount of material deposited in such a solution showed that it was probably an azo derivative, which is less toxic than dimidium (Walls and Beveridge, 1952).

The rest of the possible contributory causes of toxicity listed above have the common property of being associated with lesions in the liver. The lack of certain dietary factors, notably choline and cystine, is a well-known cause of liver disease in man and animals (Himsworth, 1947; Hartroft, 1951). The long delay in development of the signs and symptoms of liver failure in animals treated with phenan-

thridinium compounds is itself suggestive of deficiency of an essential food factor. Perhaps the drug may block biochemical reactions in which cystine, choline, or methionine are involved, and thus create deficiencies by a mechanism analogous to the folic acid deficiency produced by administration of aminopterin. Under such circumstances, the quality of the diet of the animal may make all the difference between recovery and fatal liver impairment. Evans (1948) showed very clearly that when cattle in the Sudan were transferred to an area of good grazing, the toxic effects of dimidium were very greatly reduced. Burdin and Plowright (1952) suggest that the 10-methylphenanthridinium derivatives may interfere with transmethylation; this would agree with the idea that the metabolism of choline and methionine are involved. Further evidence in support of the hypothesis was that "Ethidium," the 10-ethyl-compound corresponding to dimidium (Watkins and Woolfe, 1952), did not affect liver function or histology in doses of 3 mg./kg. However, this argument is not easy to reconcile with the other interesting observation that the 10-methyl compound phenidium (7-amino-9-*p*-aminophenyl-10-methylphenanthridinium chloride) was devoid of effects upon the liver in doses of 8 mg./kg. (Burdin and Plowright, 1952).

The presence of toxic plants in the diet could also make a contribution to the load upon the liver when a dose of a phenanthridinium compound is given. An animal might tolerate either of the noxious elements alone, but might be thrown into liver failure by their simultaneous action. The amount of photodynamic substances taken in the diet, as suggested by Wilde (1949), may explain the difference between an animal which exhibits the signs of photosensitization resulting from the accumulation of phylloerythrin in the blood, and an animal which does not.

There is as yet no sure evidence for or against the presence of the hepatotrophic virus suggested by Fiennes (1951). Such viruses are known to exist, and the almost epidemic qualities of some of the "outbreaks" of photosensitization in animals treated with dimidium lend weight to the suggestion. Further work is required, of the type already undertaken by Fiennes, to show whether or not a virus can be transmitted from photosensitized to normal animals.

Worm infestation has been observed to be a potent contributor to the toxic effects of dimidium in some parts of the Belgian Congo. It is likely that most intercurrent diseases—and few African cattle are free from them—will lower the resistance of the animals to the toxic side-effects of a drug with a narrow margin of safety.

Summary

1. A solution of the chloride (150C47), and a suspension in oil of the iodide of 2: 7-diamino-9-*p*-aminophenyl-10-methylphenanthridinium, were given subcutaneously in large doses to cattle in order to study their toxic and prophylactic effects.

2. Ten of 13 cattle treated with 2 mg./kg. or more of the drugs died between 17 and 39 days after the dose because of a toxic action which was mainly upon the liver. Fatty infiltration, centrilobular necrosis, and condensation of the reticulin framework of the liver were shown in varying degrees of severity in the animals which died. In an animal which survived, serial liver biopsies showed considerable regeneration of liver cells at the end of 11 weeks after the dose of drug.

3. The prophylactic power of the drugs was not impressive and the local reaction at the site of injection was too severe for the drug to be of value for frequently repeated doses.

4. The factors which may influence the toxicity of phenanthridinium compounds to cattle are discussed.

We are grateful to Brig. J. S. K. Boyd and Col. H. W. Mulligan for their interest and encouragement, and to Mr. E. Krog, of the Nigerian Veterinary Service, for obtaining the cattle. We wish to thank Dr. L. P. Walls for preparing the phenanthridinium compounds, and Mr. P. E. Nesbitt and Mr. H. Richards for cutting sections of a great many liver biopsy and post-mortem specimens. We also wish to thank our African assistants, especially Mr. E. Ejemuta, Mallam Tanko and his herdsmen, and Mallam Dan Boy Biu. Finally, we acknowledge the courtesy of the Director of the British West African Meteorological Services in giving us access to sunshine and rainfall records.

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ANTAGONISTIC ACTIONS AT THE NEUROMUSCULAR JUNCTION

BY

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Paton and Zaimis (1949) observed that decamethonium iodide blocks the neuromuscular junction with the same results, so far as the mechanical myogram is concerned, as tubocurarine. Each of these substances, however, is able to modify the action of the other; for instance, the injection of a small dose of tubocurarine reduces the effect of a later administration of decamethonium iodide. This observation has been confirmed by Macfarland *et al.* (1950) in man: previous "curarization" almost completely abolishes the response to decamethonium.

According to Paton and Zaimis (1949), decamethonium itself does not greatly modify the blocking properties of tubocurarine, and no real reciprocal antagonism between the two drugs exists. However, Depierre (1951) has observed an unmistakable reciprocal antagonism between flaxedil, a curare-like substance, and decamethonium. Moreover, Hutter and Pascoe (1951) have shown that suitable doses of decamethonium iodide are able to restore neuromuscular transmission after it has been blocked by tubocurarine. All these authors used cats.

The purpose of this paper is to describe our findings on the reverse phenomenon, namely the action of tubocurarine on neuromuscular block produced by decamethonium iodide. In some experiments we have used amyltrimethylammonium, the properties of which are quite similar to those of decamethonium.

Our results can be summarized as follows: tubocurarine has proved to be an excellent "deblocking agent" when administered to cats previously injected with decamethonium iodide or amyltrimethylammonium.

METHOD

The experiments were performed on cats anaesthetized with chloralose, sometimes with dial. A detailed procedure has been described elsewhere (Philippot and Dallemagne, 1951).

The tibialis anterior and soleus muscles were stimulated through the sciatic nerve every 6 sec. (supramaximal condenser discharges released by an electronic timer). The muscle twitches were recorded by means of an isometric lever coupled with Marey's drum. The movements were transmitted by a length of rubber tubing to a second drum-lever assembly equipped with a writing stylus.

The arterial pressure was recorded by means of an ordinary mercury manometer. Animals were treated with heparin.

RESULTS

Fig. 1 shows the effect of 25 μg./kg. of decamethonium iodide, injected intravenously. The injection produced a deep and long-lasting block of the neuromuscular transmission. Subsequent injection of 100 μg./kg. of tubocurarine (after

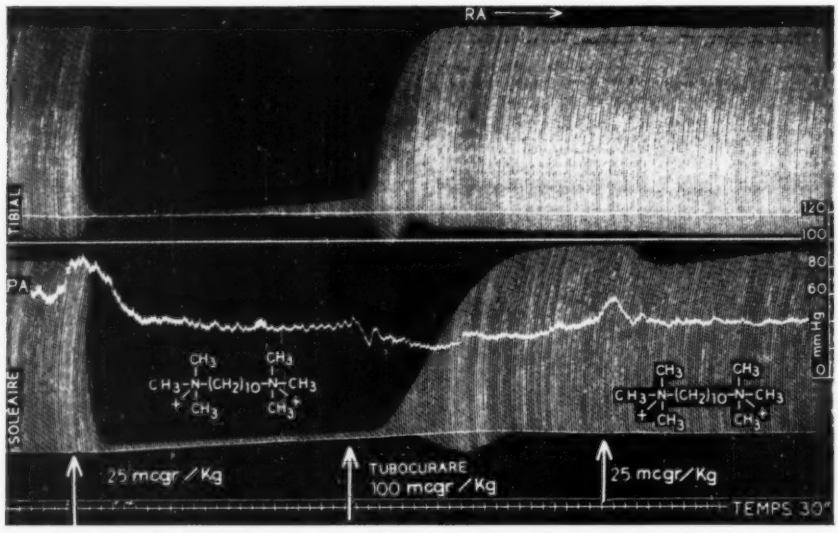

FIG. 1.—Cat (chloralose). Simultaneous records of arterial pressure, contractions of tibialis anterior and soleus. The muscles were excited with single maximal shocks at 10/sec. to the sciatic nerve. From left to right, at arrows, successively, 25 μg./kg. decamethonium iodide, 100 μg./kg. tubocurarine, and 25 μg./kg. decamethonium iodide. (On the figure, μg. is represented by mcgr.)

12 minutes) was soon followed by recovery of the junctional system. Complete restoration occurred in less than 3 min. for the tibialis anterior and in less than 9 min. for the soleus.

A fresh injection of 25 μg./kg. of decamethonium iodide did not reduce the twitches of the tibialis anterior and had only a small effect on the soleus.

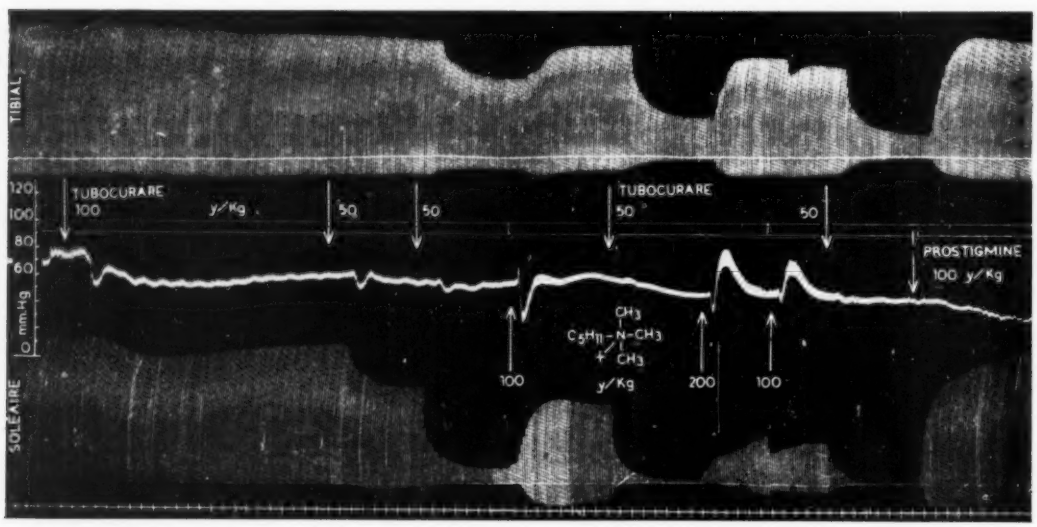

FIG. 2.—Similar preparation to that in Fig. 1. From left to right, at arrows, successively, tubocurarine, 100 μg./kg., 50 μg./kg., and 50 μg./kg.; amyltrimethylammonium iodide, 100 μg./kg.; tubocurarine, 50 μg./kg.; amyltrimethylammonium, 200 μg./kg., 100 μg./kg.; tubocurarine, 50 μg./kg.; and prostigmine, 100 μg./kg. (On the figure, μg. is represented by γ.)

After the first injection of decamethonium iodide, the respiratory movements (not recorded in Fig. 1) were not significantly depressed in spite of the marked paralysis of the hind limbs. After the injection of tubocurarine, the amplitude of the respiratory movements decreased, while normal neuromuscular transmission was being restored in the limb. Before long, spontaneous respiration stopped altogether.

Amyltrimethylammonium, like butyltrimethylammonium (Philippot and Dallemagne, 1951), is antagonistic to curare. Fig. 2 shows the effect of repeated injections of tubocurarine: it is well known (Paton and Zaimis, 1949) that the soleus is more sensitive to the paralysing action of tubocurarine than is the tibialis anterior.

When amyltrimethylammonium was injected during this period of decreased twitch tension, there was a complete restoration of the neuromuscular transmission in the tibialis anterior and a partial recovery of the soleus.

A further injection of tubocurarine was more efficient, and the decurarizing action of amyltrimethylammonium was now smaller. A third injection of this drug no longer increased the twitch tension, but reduced it transiently. The junctional system was still sensitive to tubocurarine. Neostigmine was able to restore neuromuscular transmission completely.

The successive deblocking and blocking actions of tubocurarine are clearly shown in Fig. 3. In this experiment, neuromuscular block had been produced by the

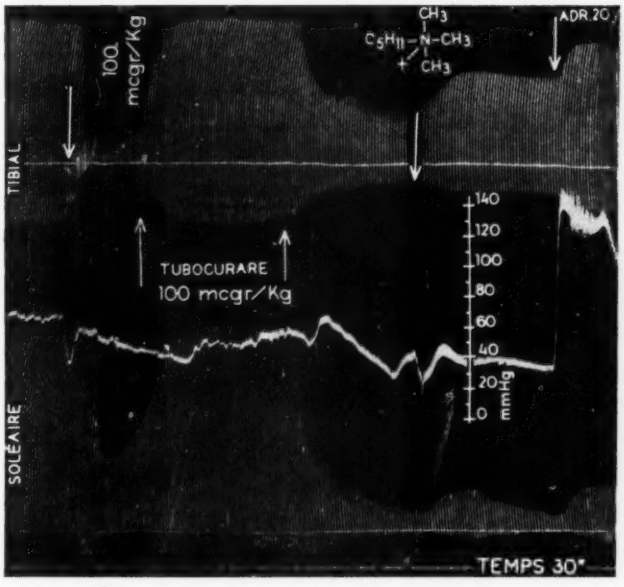

FIG. 3.—Similar preparation to that in Fig. 1. From left to right, at arrows, successively, amyltrimethylammonium, 100 μg./kg.; tubocurarine, 100 μg./kg., 100 μg./kg.; amyltrimethylammonium, 100 μg./kg.; and adrenaline, 20 μg./kg. (On the figure μg. is represented by mcgr or γ.)

injection of amyltrimethylammonium. There was a definite tendency to spontaneous recovery; but the first injection of tubocurarine undoubtedly accelerated the increase of twitch tension. The second injection had an opposite effect, i.e. true curarization. Subsequent injection of amyltrimethylammonium was able partly to restore neuromuscular transmission in the tibialis anterior. There was only a small effect on the soleus. Adrenaline had the same action.

Discussion

In a previous report (Philippot and Dallemagne, 1951) we described the reciprocal antagonism between tubocurarine and butyltrimethylammonium. Our present findings show a similar antagonism between tubocurarine and amyltrimethylammonium or decamethonium. This suggests that decamethonium iodide and the lower homologues of alkyltrimethylammonium salts exert their paralysing effects through a somewhat similar mechanism.

The most spectacular phenomenon was the deblocking action of tubocurarine when paralysis had been induced by the so-called acetylcholinomimetic compounds, such as decamethonium iodide and amyltrimethylammonium. The optimal doses required to reveal this antagonism are equi-active: in other words, they exhibit the same blocking activity when used alone.

After injection of tubocurarine and decamethonium iodide (or amyltrimethylammonium), it is possible to obtain a state of functional disequilibrium at the neuromuscular junction ; either true curarization may occur or a paralysis of the acetylcholinomimetic type.

The " deblocking " action of tubocurarine is very strong, while the " decurarizing " properties of decamethonium iodide are definitely weaker and more irregular. This difference may be due to the fact that decamethonium does not act only on the motor endplate itself, but also produces a depolarization of the adjacent muscle fibres.

All muscles are not equally sensitive to the drugs mentioned. The soleus is more sensitive than the tibialis anterior to the action of tubocurarine ; the reverse holds for decamethonium iodide (Paton and Zaimis, 1949) and amyltrimethylammonium.

These reasons (choice of the optimal dose and unequal sensitivity of the muscles) explain why repeated injections in the same animal generally result in the disappearance of the antagonism described, at least at the level of one of the two muscles studied.

It must also be noted that tubocurarine is not antagonized by decamethonium or other acetylcholinomimetic compounds (and vice versa) when the experiments are performed on dogs. Decamethonium is poorly active in the latter species and blocks neuromuscular transmission in this animal apparently by the same mechanism as tubocurarine does: its blocking effects are enhanced by tubocurarine and alleviated by adrenaline and neostigmine (Dallemagne and Philippot, 1952).

Summary

These experiments have disclosed the powerful deblocking effects exerted by tubocurarine on neuromuscular block produced by decamethonium iodide or amyltrimethylammonium in the cat.

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THE HORMONES OF THE ADRENAL MEDULLA AND THEIR RELEASE*

BY

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The evolution in recent years of sensitive tests for noradrenaline has led to a revival of interest in the adrenal medulla, especially because the medullary hormones were more likely to show differences in the extent of N-methylation in different animals and under different conditions than nerve sympathin, of which almost invariably very little is methylated. Among numerous recent investigations by several workers two in particular have a bearing on the present work. The liberation of adrenaline and noradrenaline from the adrenals of cats, when the splanchnic nerves were stimulated, was studied by Gaddum and Lembeck (1949), using the method of parallel quantitative assays; they pointed out, however, that the errors of the tests used were so large that convincing evidence was only obtained of the presence of a mixture of substances and not of their relative proportions. Adrenaline and noradrenaline in the adrenals of rats injected with insulin were estimated by Burn, Hutcheon, and Parker (1950). Their method involved the simultaneous recording of the blood pressure and of the contractions of the normal nictitating membrane of the cat. The latter test object gave a measure of the proportions of the two amines when various equipressor mixtures were injected.

In the present work studies were made of the changes in the adrenaline and noradrenaline content of the adrenals of rats under the influence of three drugs known to cause a release of the adrenal medullary hormones: insulin (Cannon, McIver, and Bliss, 1924), morphine (Elliott, 1912), and β-tetrahydronaphthylamine (Mutch and Pembrey, 1911). Experiments were also carried out on cats in which the adrenals were secreting spontaneously, or were being stimulated through the splanchnic nerves or by the injection of acetylcholine or potassium. The amines were estimated in the glands and in the plasma of the effluent blood.

METHODS

Rats of both sexes, weighing 150–200 g., were used. For the insulin experiments they were fasted overnight (17 hours) and during the course of the experiments. Groups of 4–8 rats were divided into two equal subgroups. One subgroup was injected subcutaneously with a drug and the other subgroup served as a control. The drugs and doses per 100 g. of rat were: soluble insulin (Burroughs Wellcome and Co. 20 units/ml.), 1 unit; morphine hydrochloride (Macfarlane), 2 mg. in a freshly made solution containing 2 g./100 ml.; tetrahydro-β-naphthylamine carbonate (B.D.H.), 7.5 mg. in a

* This work formed part of a thesis for the degree of Ph.D. of Edinburgh University.
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freshly made slightly acid solution containing 2 g./100 ml. Both injected and control animals were killed together by a blow on the head at various times after administration of the drugs. The adrenals were quickly removed and weighed. Both glands of each animal were placed in 0.5 ml. of 0.15 N-HCl in a "Pyrex" centrifuge tube. When all the glands from the particular experiment had been collected they were ground up in each tube with the assistance of a little acid-washed sand. The extracts were then prepared for application to a cylinder of filter paper for chromatography as described by Crawford and Outschoorn (1951).

Cats, weighing over 2 kg. if possible, were anaesthetized with ether and chloralose and eviscerated. Collections of blood were made in a similar manner to that described by Gaddum and Lembeck (1949) through a cannula in the inferior vena cava by transferring a clip on that vessel from below the site of entry of the renal veins to just below the liver. The renal vessels were always tied, but the kidneys were left *in situ*. In some experiments, in which the left adrenal vein joined the left renal vein, only the left gland was stimulated. The kidneys were still tied off, but the collecting cannula was tied into the left renal vein and the blood shunted through a rubber tubing by-pass into an external jugular vein. Adrenal venous blood was then collected through a T-piece in the rubber tubing by transferring a clip from a piece of rubber tubing on a stem of the T to the rubber tubing on the appropriate limb of the T. The splanchnic nerves were cut leaving a good peripheral length for electrical stimulation. Drugs were injected through a cannula in the stump of the coeliac artery, which was at other times occluded by a clip. The animals were heparinized (100 units/kg.) and the carotid blood pressure was recorded with a mercury manometer. The splanchnic nerves were stimulated with an alternating current from a Ritchie-Sneath stimulator, using 5–7.5 volts for platinum electrodes or 40–50 volts for a Collison's electrode (in which the nerve was surrounded by Locke's solution). Acetylcholine (100 mμg./ml. in saline) or potassium chloride (1 g./100 ml. water) was injected in volumes of 0.5–1 ml. either slowly over 5–10 min. or in a moment. Samples of adrenal venous blood were taken during control periods and when the glands were stimulated. Samples of arterial blood were sometimes taken as well. The blood was received into ice-cooled graduated centrifuge tubes, centrifuged (3,000 r.p.m. for 10 min.), and the plasma pipetted off and stored in ice until further treatment. Two types of experiment were performed. In the first series the periods of collection and stimulation were long (5–10 min.) and were accompanied by a progressive fall in the cat's blood pressure. The substances in the plasma were estimated by parallel assays on the rat's uterus, the rat's colon, and the rabbit's ear (Gaddum, Peart, and Vogt, 1949). In the second series the periods of collection were short (30–60 sec.) and the fall in blood pressure was much less; sometimes it was prevented by infusing blood from another cat between samples. Usually, however, it was sufficient to leave the animal for about 5 min. to let the blood pressure return to its original height after collection of a sample. The samples of plasma were subjected to preliminary tests on the rat's uterus and then prepared for paper chromatography.

A measured volume of plasma (up to 2 ml.) was added dropwise to 6 volumes of acid alcohol (0.1 per cent (v/v) conc. HCl in ethanol) and chilled in ice for 30 minutes. The tubes were centrifuged (3,000 r.p.m. for 5 min.) and the supernatant fluid was transferred to a 50 ml. round-bottomed Quickfit and Quartz flask; the precipitate was washed with 2 ml. acid alcohol which was chilled for 10 min., centrifuged (3,000 r.p.m. for 3 min.), and added to the first fraction. The cooled fluid was evaporated *in vacuo* at 55°–60° C. (external temperature). The residue was transferred with 5 washings of 1 ml. each of acid alcohol to a 10 × 1 cm. hard glass centrifuge tube which was chilled in ice for 15 min. and centrifuged (3,000 r.p.m. for 3 min.). The supernatant fluid was transferred to another 50 ml. round-bottomed flask, the residue being washed with another 1 ml. of acid alcohol which was chilled for 5 min., centrifuged (3,000 r.p.m. for

3 min.) and added to the fractions in the flask. The pooled fluid was evaporated *in vacuo* at 55°–60° C. (external temperature). The residue was leached with 0.7 ml. acid alcohol, which was applied to a paper cylinder for chromatography, and then with a further 0.2 ml. which was also applied.

In some experiments extracts of the adrenal glands were prepared for chromatography. In these experiments one gland was removed before cannulation of the inferior vena cava as a control while the other was left for electrical stimulation of the splanchnic nerve. This was carried out continuously for 1 hour during which blood samples were taken at half-hourly intervals. At the end of the period the remaining gland was removed. Each gland was weighed soon after removal, placed in 10 ml. of 0.15 N-HCl in a mortar, and ground. When well homogenized, 1 ml. of the liquid was pipetted off and added to 10 ml. acid alcohol. The preparation of the extract for chromatography was similar to that for the whole liquid containing a pair of rat's adrenals, save that the precipitation was aided by chilling in ice instead of leaving the sample at room temperature, and the centrifugings were for a shorter time (5 min.) at a higher speed (3,000 r.p.m.). The final leachings, with 0.7 and 0.2 ml. acid alcohol, were applied to a paper cylinder.

The application of the acid alcohol extracts to the paper, the development of the chromatogram with phenol as solvent, the elution of the appropriate strips of paper containing adrenaline and noradrenaline, and the assay of these amines in the eluates with the rat's blood pressure preparation were as described by Crawford and Outschoorn (1951), with the difference that the rats used for testing were treated with atropine and hexamethonium, which lowered their blood pressure and so decreased the effects of depressor substances in the samples. Atropine sulphate (100 μg./100 g.) was given intravenously slowly over about 2 min. and the animal left for about 10 min. to recover. Hexamethonium (C6) bromide (1 mg./100 g.) was then given intravenously, slowly over 2–3 min. The blood pressure usually fell to about 50 mm. mercury, and the rat was used immediately for assays. After about 0.75–1 hour the blood pressure had often risen to about 100 mm. mercury and this was accompanied by a reduction in the sensitivity and discrimination of the preparation. It was sometimes necessary to lower the rat's blood pressure again by repeating the injection of C6 in about double the dosage.

Experiments on rats.—There were several possible ways of presenting the results of the rat experiments. The concentrations of the amines could be expressed in terms of gland weight (mμg./mg.) or of body weight of rat (μg./100 g.). In the insulin experiments the effects of starvation and of the drug had to be considered. Vogt (1947) reported the influence of these on the weights of the adrenals. Fig. 1 shows the changes undergone by the mean weights of the glands in terms of body weight of rat in the present insulin experiments. Both control and injected groups agreed fairly closely in showing a fall in weight up to about 6–8 hours and an increase in weight after 16 hours. Owing to these changes the concentrations of adrenaline and noradrenaline in all the rat experiments were expressed in terms of body weight of rat, which presumably varied much less.

Wide variations were noticeable in the concentration of the amines in the adrenals of the control animals. Analyses of variance were made of the estimates of adrenaline and noradrenaline in the control rats of (*a*) the insulin experiments and (*b*) the morphine and tetrahydronaphthylamine experiments taken together. This was because the rats of the insulin experiments were fasted overnight while those of the other experiments were fed. For adrenaline and for noradrenaline there was in both series a significant variation between the control values from day to day

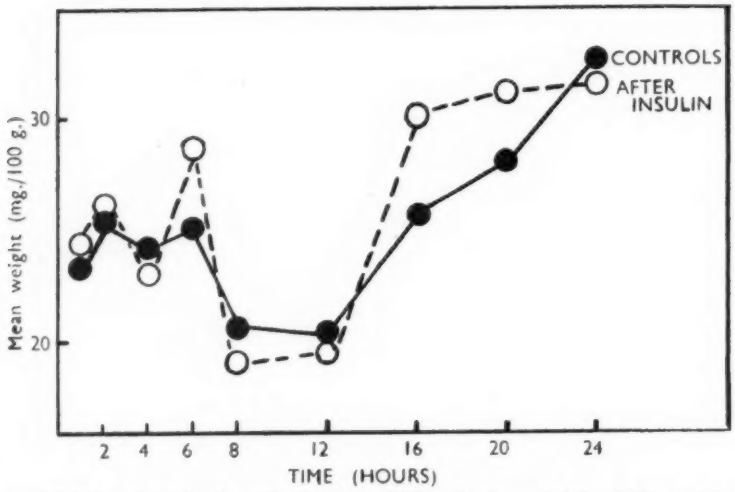

FIG. 1.—Weight of rats' adrenals (mg. per 100 g. body weight). Effect of fasting.

and at different times after the commencement of the experiments. On account of these variations among the controls it was decided that the concentration of the amines in each injected animal should be calculated as a percentage of the mean amount in the group of control rats treated in the same way and killed at the same time. A mean value for the amount of each amine found after a particular time of action of a drug was then calculated from the individual percentages. Table I

TABLE I

In this Table *n* = no. of injected rats; Adr. = adrenaline; Nor. = noradrenaline

Hours after injection	Calculated mean concentrations of pressor amines in rats' adrenals (as percentages of means of control animals) after											
	Soluble insulin			Morphine hydrochloride						Tetra-hydronaphthyl-amine		
				Single dose			Repeated doses					
	n	Adr.	Nor.	*n*	Adr.	Nor.	*n*	Adr.	Nor.	*n*	Adr.	Nor.
1	5	70	98	6	94	99						
2	21	99	147	3	137	158				3	68	81
4	5	54	112	3	88	64	6	43	67	2	66	131
5							3	59	131			
6	5	56	100									
8	5	55	87	3	79	105				3	25	81
12	5	22	52	3	111	188						
13										1	53	115
16	5	15	72	3	84	85				3	90	76
20	5	33	80	3	99	156						
24	4	28	112									
Range of mean conc. in controls (μg./100 g. Adr.		13.75—31.82		8.06—13.96			11.80—16.77			11.57—15.21		
Range of mean conc. in controls (μg./100 g. Nor.		0.95—3.48		0.94—2.86			2.17—3.33			1.82—3.22		

shows the results calculated in this way. However, when tests of significance were made, the absolute concentrations of each amine in the group of the injected rats was compared with those of the controls for the particular time of action of the drug even though that included experiments carried out on different days.

Adrenaline.—Insulin produced a significant depletion of this amine. In 12 hours the injected animals had about 20 per cent of the amount present in the controls and in 16 hours only 10–20 per cent. Thereafter and up to 24 hours a tendency towards recovery was noticeable. With tetrahydronaphthylamine the depletion was quicker. About 25 per cent of the adrenaline was left in 8 hours and recovery was almost complete by 16 hours. After only one injection of morphine there was no appreciable depletion of adrenaline over 20 hours' observation. A transient but significant depletion to 43 per cent of the original level was achieved with four injections of the drug at hourly intervals, the animals being killed 4 hours after the commencement. After 5 hours the depletion was less.

Noradrenaline.—The amounts of noradrenaline did not follow the trend of the methylated amine. Neither morphine (single or repeated injections) nor tetrahydronaphthylamine produced any lowering of the content of noradrenaline below the range of the control animals. Of the three agents (in the particular dosages employed) the most powerful and longest lasting effect was produced by insulin. Even this produced only a transient, non-significant depletion of less than 50 per cent of the noradrenaline at 12 hours, while recovery was complete in 16–20 hours. On the other hand, 2–4 hours after the injection of the drugs there was an indication that the noradrenaline content was increased 30–50 per cent above that of the controls. Although this increase was small, transient, and only just significant ($P = 0.05$) in the insulin injected rats, a similar trend was observed in the experiments with the other two agents. Fig. 2 shows the changes undergone by the adrenaline and nor-

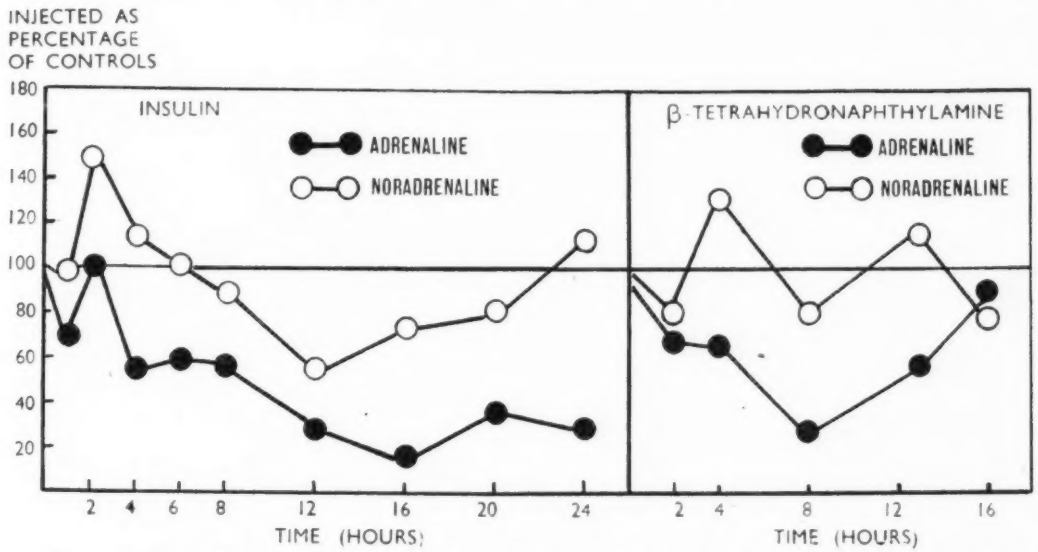

FIG. 2.—Amounts of adrenaline and noradrenaline in rats' adrenals (mg. per 100 g. body weight) expressed as percentages of figures for control rats. Effects of insulin and β-tetrahydronaphthylamine.

adrenaline in the adrenals of the injected animals. A figure for the morphine experiments was not constructed owing to the relatively small effects of that drug.

Experiments on cats.—The results of parallel assays on samples of adrenal venous plasma from the first series of experiments on cats are shown in Table II. In some

TABLE II

Cat: Estimates of adrenaline and noradrenaline in adrenal venous plasma by parallel assays R_u = dose ratio of noradrenaline to adrenaline required for equal responses on uterus. R_c = dose ratio of noradrenaline to adrenaline for equal responses on colon

Exp. No.	Adrenaline equivalents (mμg./ml.)					Calculated concn. (mμg./ml.)				
	Serial samples	Assay with uterus	R_u	Assay with colon	R_c	Adrenaline	Noradrenaline	% Adr.		
55	Control	<2.5	150	<50	0.5	<2.3	<24	—		
	Ach.	300	150	200	0.5	299	(−50)	100		
	Ach.	375	150	375	0.5	374	0	100		
	Control	75	75	87	0.5	75	6	93		
	Control	12	75	<50	0.5	12	<19	<39		
	KCl	3,000	75	3,000	0.5	2,979	0	100		
	Control	500	75	1,500	0.5	490	500	49		
57	Control	75	160	125	2.5	73	125	37	Assay with rabbit's ear	R_e
	Ach.	562	160	750	2.5	550	470	54		
	Ach.	1,200	160	1,500	2.5	1,177	750	61		
	Control	1,250	160	1,750	2.5	1,223	1,250	49	3,000	3
58	Control	30	175	<5,000	0.75	≥10	<3,727	—		
	Ach.	4,876* (s.e. 12.4%)	175	790* (s.e. 40.1%)	0.75	4,873	(−3,064)	100	*Statistical	
59	Control	<5	150	Inter.	0.75	<5	—	—		
	Electr.	3,200	150	9,333	0.75	3,153	4,600	41		
	Control	1,500	150	2,000	0.75	1,490	375	80		
	Arterial	Inter.	150	Inter.	0.75	—	—	—		
60	Control	2.5	250	<250	3	(−0.5)	<742	—	Assay with rabbit's ear	R_e
	Electr.	2,500	250	2,000	3	2,476	(−1,500)	100		
	Control	562	150	1,500	0.5	558	469	54		
	Arterial	<2.5	250	<250	3	—	<742	—	10,000	1.87
61	Control	15	37	<500	0.75	≥5	<364	—		
	Electr.	6,000	37	6,000	0.75	5,880	0	100		
	Arterial	Inter.	37	Inter.	0.75	—	—	—		
62	Control	7.5	75	<250	0.37	≥6.2	<92	—		
	Electr.	1,500	75	3,000	0.37	1,485	555	73		
63	Control	7.5	100	<500	1	≥2.5	<492	—		
	Electr.	1,000	100	1,000	1	900	0	100		
	Ach.	12,360* (s.e. 16.8%)	100	17,866* (s.e. 25.7%)	1.5	12,092	8,259	59	*Statistical	
64	Control	10	75	<500	0.75	≥5	368	—		
	Electr.	6,480* (s.e. 28.3%)	110*	1,693* (s.e. 51.2%)	0.7*	6,469	(−3,351)	100	*Statistical	
	Ach.	19,540* (s.e. 27.9%)	110*	10,720* (s.e. 36.7%)	0.7*	19,472	(−6,174)	100		

assays the complete statistical procedure adopted by Gaddum and Lembeck (1949) was used, but significant amounts of noradrenaline were not found. The release of large amounts of active substances on stimulation of the adrenal medullae is evident. Generally stimulation samples taken after control samples showed an apparent increase in the percentage of adrenaline as well, whereas control samples taken after stimulation samples showed an apparent decrease both in total activity and in the percentage of adrenaline. Accordingly the hormones released must have consisted mainly of adrenaline. A better idea of the proportions of the two amines was afforded by the second series of experiments. Assays were carried out after chromatographic separation of adrenaline and noradrenaline, and the results are given in Table III. The percentage of adrenaline in the control samples could not be calculated from the rat's blood pressure assays only. In one experiment (No. 68) the percentage was estimated as 5 per cent from

TABLE III

Cat: Estimates of adrenaline and noradrenaline in adrenal venous plasma separated by chromatography with the rat's blood pressure preparation

Comparison of different methods of stimulation: Control = unstimulated. Electr. = electrical stimulation (see text). Ach. = Acetylcholine injection. KCl = Potassium chloride injection.

Expt. No.	Sample: control or method of stim.	Adrenaline equivalent by direct assay with the rat's uterus mμg./min.	Estimates of separated amines with the rat's blood pressure mμg./min.		% Adr.
			Adrenaline	Noradrenaline	
65	Control	—	<551	412	<57
	Electr.	—	1,332	1,248	52
	Ach.	—	3,268	612	84
	KCl	—	4,960	1,550	76
	Ach.	—	4,799	2,800	63
	Electr.	—	2,400	600	80
66	Control	180	<300	<375	—
	Electr.	720	775	<186	>81
	Ach.	8,000	5,000	<625	>89
67	Control	<10	<1,000	<500	—
	Electr.	2,800	4,375	3,500	56
	Ach.	4,560	7,600	5,700	57
	KCl	5,000	10,000	5,000	67
	Ach.	1,440	3,000	1,500	67
	Electr.	—	3,256	4,888	40
68	Control	19	<950	357	<73
	Electr.	1,001	600	581	51
	Ach.	6,400	3,240	8,000	29
	KCl	2,855	2,800	2,500	53
	Ach.	4,800	3,528	1,500	70
	Electr.	925	1,388	462	75
69	Control	2.6	<525	<262	—
	Electr.	1,040	975	455	68
	Ach.	1,600	1,250	625	67
	KCl	4,400	3,300	2,475	57
	Ach.	2,666	2,500	1,500	62
	Electr.	3,466	3,250	1,950	62

the result with rat's uterus. In two experiments (65 and 68) there was assayable noradrenaline in the control plasma. There was an increase in total activity on stimulation which was once (Exp. 68) accompanied by an increase in the percentage of adrenaline. The amines could both be estimated accurately in plasma collected during stimulation, and the percentage of adrenaline was practically the same after all three forms of stimulation. In a few experiments attempts were made to investigate the relation of the amounts of adrenaline and noradrenaline in the adrenal glands to those in the venous plasma. The results are shown in Table IV. In two experiments (70 and 71) the left adrenal was removed as a control and the right splanchnic nerve was stimulated. This proved unsatisfactory, probably owing to partial occlusion of the right adrenal vein when the inferior vena cava was clipped just below the liver. In the remaining experiments the right adrenal was removed as a control and the left was stimulated through the splanchnic nerve, with more satisfactory results. Tests on samples of plasma showed that after one hour's continuous stimulation the proportions of adrenaline and noradrenaline did not change.

TABLE IV

Cat: Estimations of adrenaline and noradrenaline in adrenal glands and adrenal venous plasma: separated by chromatography and assayed with the rat's blood pressure

Effect of continuous stimulation. Control gland removed before stimulation. Stimulated gland removed at end of period of stimulation.

Exp. No. (Wt. of cat)	Stim. for (hr.)	Adrenaline equiv.: Rat's uterus	Separated by chromatography, assayed with the rat's blood pressure						
			Venous plasma (mμg./min.)			Adrenal glands (μg./gland)			
			Adrenaline	Noradrenaline	% Adr.	Weight	Adrenaline	Noradrenaline	% Adr.
70 (2.85 kg.)	Control	<2	<48	<21	—	Control (142 mg.)	150	100	67
	0	27	<62	15	<80				
	½	30	<62	63	<50	Stim. (144 mg.)	75	50	67
	1	27	208	110	65				
71 (3.4 kg.)	Control	<2.8	<79	<25	—	Control (264 mg.)	75	25	75
	0	36	113	74	60				
	½	75	188	75	71	Stim. (246 mg.)	66.7	14.6	82
72 (4.2 kg.)	Control	<8.5	<36	70	<34	Control (238 mg.)	100	15	87
	0	360	240	450	35				
	½	315	275	275	50	Stim. (226 mg.)	75	10	87
	1	286	262	131	67				
73 (2 kg.)	Control	>>180	841	599	58	Control (120 mg.)	100	37.5	73
	0	800	333	500	40				
	½	75	125	62	67	Stim. (108 mg.)	50	25	67
	1	87	87	54	62				
74 (3.6 kg.)	Control	<9	<30	<15	—	Control (190 mg.)	100	75	57
	0	256	426	426	50				
	½	408	303	454	40				
	1	90	100	150	40	Stim. (218 mg.)	67	50	57
Mean depletion (per cent) after 1 hour							40	46	

In the same period there was an appreciable depletion of both amines to about the same extent (less than 50 per cent), so that the percentage of adrenaline in the stimulated gland was the same as in the control gland removed earlier. The percentage of adrenaline in the plasma was generally less than in the glands.

Discussion

The amounts of sympathomimetic amines in the adrenals of an animal are determined by a balance between utilization and synthesis. From the results for the rat adrenals as well as from those for the cats it would appear that with a sufficiently powerful and prolonged stimulus the utilization of adrenaline increasingly outpaces synthesis, with the result that the glands are depleted of that amine. In the rat adrenals the depletion of adrenaline was most marked in the insulin experiments, less pronounced in the tetrahydronaphthylamine experiments, and could only just be demonstrated in the experiments in which repeated doses of morphine were given. In the cats' adrenals even after a comparatively short stimulation period of 1 hour some depletion of adrenaline was evident. The amounts of noradrenaline showed different changes in the two species of animals. Under the conditions of the rat experiments the noradrenaline level kept within the normal range, although there was a tendency to depletion in the insulin-injected rats. This may be because there was either no output of noradrenaline or an output with which synthesis could keep pace. If the latter explanation is correct, the rat is perhaps better able to synthesize noradrenaline than adrenaline when its medullary resources are heavily taxed. On the other hand, in the experiments with cats the adrenals were depleted of noradrenaline to the same extent as of adrenaline. When the adrenals are stimulated there is an output of noradrenaline into the blood in the cat, but it is less than that of adrenaline. The parallel depletion of the two amines in spite of the smaller output of noradrenaline in relation to adrenaline shows that the cat has no special ability to synthesize noradrenaline quickly.

In the rat adrenals, owing to the much greater utilization of adrenaline than of noradrenaline which occurred after prolonged stimulation of the adrenal medulla, there tended to be a relative increase in the proportion of noradrenaline, which occurred 12 hours after insulin and 8 hours after tetrahydronaphthylamine. There was no evidence of an absolute increase of noradrenaline at these times. Burn, Hutcheon, and Parker (1950) in their experiments on rats showed that after 0.2 unit of insulin per 100 g. rat there was a 50 per cent depletion of the total amount of medullary hormones in the adrenals after 8 hours. At this time there was also a decrease in the percentage of adrenaline. They concluded that an increase in the relative amount of noradrenaline, as determined by their method of assay, was due to the process of methylation being slower than the processes by which fresh noradrenaline was accumulated, after the adrenals had been active for 4–6 hours. In their experiments the pooled adrenals of several rats were extracted and tested. In the present series the amines present in the pair of adrenals of each animal were estimated individually and separately. There was evidence of an absolute increase of noradrenaline in the rat adrenals 2–4 hours after injection of the drugs. During this increase of noradrenaline the amounts of adrenaline were hardly less than in the controls. The tendency for the earliest response of the adrenals of rats to be an increased production of noradrenaline, even while the

demand for adrenaline could still be met, is in conformity with the other evidence suggested above of the power possessed by the rat to synthesize noradrenaline. This early increase in the absolute amount of noradrenaline has also been reported by West (1951) in rabbits 2 hours after the injection of insulin. Assayable amounts of noradrenaline were found, which were not detectable at other times.

The percentage of adrenaline in the cat adrenals in these experiments showed no change during one hour's stimulation, whereas on repeated or prolonged stimulation Bülbring and Burn (1949a) and West (1949) noticed a fall in the percentage of adrenaline. The former workers had some evidence that this change was dependent on methionine deficiency in the animals' diets. The differences in the cat experiments reported here may be related to the intensity of the stimulus as well as to the fact that the depletion of total amines obtained was relatively small.

There was some difficulty in the experiments on rats in producing a depletion of adrenaline with morphine. This contrasts with Elliott's (1912) experiments on cats, where 20 mg. of morphine depleted the adrenals to 15 per cent of the control adrenaline content in 8 hours. With 2–3 c.c. of a 2 per cent (w/v) solution of tetrahydronaphthylamine only about 50 per cent of the adrenaline was depleted in the same time. Both drugs were used in the rats at about twice this dose per kg., but only tetrahydronaphthylamine produced an effect on the stores of hormone in the adrenal medulla of the rat. Evidently morphine seems more efficient in depleting the adrenals of cats than of rats, whereas the opposite is true for tetrahydronaphthylamine.

Since a certain degree of hypoglycaemia after insulin is essential for the initiation of the stimulus to the adrenals (Cannon, McIver, and Bliss, 1924), a check was made on the relationship between the changes in the amounts of the medullary amines and the blood sugar level. Blood sugar estimations (Hagedorn–Jensen method) gave mean values (of 3 rats in mg./100 ml. of blood) of 88 at the commencement, 54 in 2 hours, 51 in 4 hours, and 89 in 5 hours after insulin. The depletion of adrenaline appeared to have continued long after the blood sugar level had returned to normal.

The long duration (more than 24 hours) of the depletion of the adrenaline in the glands after insulin was like that observed in dogs injected with eserine by Edmunds and Smith (1932), who found only 10–20 per cent of the normal adrenaline after 24 hours and less than normal after 48 hours.

In the second series of experiments on cats, blood was injected in order to maintain the blood pressure at the same level at the commencement of each sampling so as to avoid the influence of haemorrhage on the adrenals as much as possible. In addition the types of stimulation were repeated thus: electric stimulation, acetylcholine, KCl, acetylcholine, electric stimulation, in each experiment. This was to prevent any change in the amounts of the amines or in the proportion of adrenaline due to a general shift caused by the condition of the animal passing unnoticed.

No differences were observed between the three methods of stimulation on the proportion of adrenaline to noradrenaline in the medullary hormones released in the cat. Either the method of investigation was not sensitive enough to detect such differences or the proportion of adrenaline depends on other factors, such as the supplies of noradrenaline available to the glands. Bülbring and Burn (1949b) found that noradrenaline added to the perfusing fluid of a dog's isolated adrenal resulted in an increase of adrenaline in the outflow.

The high output of active substances in the control plasma in one experiment (73) can be explained by an unintentional mechanical stimulation caused by respiratory movements displacing the electrode which was usually fixed in position before any samples (control or stimulation) were taken.

The suggestion of Meier and Bein (1948) that there was probably a constant discharge of noradrenaline from the resting adrenals is supported by the finding that this amine could be detected in the venous plasma of the control samples in some of the cat experiments. It is not likely that its presence was due to stimulation in operation, because it was not accompanied by a large amount of adrenaline. The instances in which it was possible to measure the percentage of adrenaline in control samples are too few to decide whether it is normally low in the conditions of these experiments. The experiments showed throughout that stimulation by any of the methods used caused a release of a mixture in which adrenaline predominated. It is, however, still doubtful whether a constant secretion of noradrenaline is part of the normal physiological function of the adrenals.

Summary

1. Insulin, morphine, and tetrahydro-β-naphthylamine were injected into groups of rats. All three agents produced significant depletion of adrenaline in the adrenals after some hours. No significant depletion of noradrenaline occurred, although there was a tendency to depletion with insulin.

2. At the time of maximum depletion of adrenaline there was a relative increase of noradrenaline and thus a reduction in the percentage adrenaline content. An absolute increase of noradrenaline occurred within the first few hours of stimulation, at which time the amounts of adrenaline were still normal.

3. The adrenals of cats were stimulated through the splanchnic nerves electrically, and by acetylcholine and potassium. Small amounts of adrenaline and sometimes larger amounts of noradrenaline were found in the venous plasma from the resting gland. Stimulation caused the release of large amounts of adrenaline and smaller amounts of noradrenaline. The proportion of adrenaline to noradrenaline did not appear to depend on the method of stimulation, nor did it alter after continuous electrical stimulation for 1 hour.

4. In the same period the adrenal gland was depleted of less than 50 per cent of its pressor amines, but of both adrenaline and noradrenaline to the same extent. In this it differs from rat adrenals, where only adrenaline was depleted appreciably.

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THE NATURE OF SYMPATHIN RELEASED IN THE RABBIT'S EAR*

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It is now generally accepted that most mammalian adrenergic nerves release a mediator consisting largely of noradrenaline, which may be admixed with a varying but small proportion of adrenaline. The adrenergic nerves in the rabbit's ear were investigated by Gaddum and Kwiatkowski (1938, 1939) and Gaddum, Jang, and Kwiatkowski (1939), and found to release adrenaline. In view of the fact that no evidence was found for the release of noradrenaline these nerves have been quoted as exceptions to the general rule (Euler, 1950). Since there are now more sensitive and specific tests for the identification of the adrenergic mediators it was decided to reinvestigate the nature of those released in the rabbit's ear.

METHOD

A large rabbit (over 2.5 kg. where possible) was anaesthetized with pentobarbitone sodium ("Nembutal" Abbott, containing 1 grain per ml.) in a dosage of 1.0 ml./kg. intraperitoneally, repeated every 15 minutes till anaesthesia was complete (0.5–1.5 hour).

The animal was fixed in a supine position on a warmed table. The trachea was quickly cannulated and artificial respiration instituted. The common carotid artery was identified and followed headwards to its terminal branches. The aim of the dissection throughout was to identify the arterial branches rather than to clean the structures surrounding the vessels. All the branches of the common carotid artery were tied off, including the terminal ones, but excluding the artery to the ear which arises laterally about the level of the superior cervical ganglion. The tied vessels were not divided.

The cervical sympathetic trunk was traced up to the superior cervical ganglion and the fine post-ganglionic fibres, running laterally, carefully dissected for 0.2–0.5 cm. Where these post-ganglionic fibres were in close proximity to the auricular artery no attempt was made to separate them from the vessel, but where the anatomy permitted, the nerves were cleared separately. The great auricular vein was freed. For identification, a loose ligature was placed around its union with the external jugular vein. The common carotid artery was then cannulated and the perfusion with salt solution commenced. The arterial cannula was usually of a simple straight type, but where the preparation was to be used later for assays (Gaddum, Peart, and Vogt, 1949) the special cannula described by Gaddum and Kwiatkowski (1938) was used. As soon as the salt solution was flowing into the ear the external jugular vein was cut with scissors to allow the perfusate to flow out freely. The tissues of the neck were completely severed with a scalpel as low down as possible, and the vertebral column was cut with bone forceps, thus decapitating the animal. In order to prevent leakage, the tissues of the neck were tied with a stout cord at a level below the arterial cannula and the cut in the jugular

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vein. Any possible leaking vessels were thus compressed against the cervical vertebrae. The vertebral canal was blocked tightly with plasticine and a cork.

A straight glass cannula was then inserted into the great auricular vein through the orifice in the jugular vein and tied therein. The reservoir containing the perfusing fluid was fixed about 2–3 m. above the preparation. The fluid passed through a 3–4 cm. length of capillary tubing fitted with a by-pass of rubber tubing with a clip. When the clip was closed the only passage available was through the capillary. The by-pass enabled the preparation to be washed out. The clip was generally opened slightly when the perfusion commenced, but tightened when the effluent from the ear no longer contained evident traces of blood. A water manometer of small-bore glass tubing was interposed between the capillary tube and the arterial cannula. The apparatus was similar to that devised by Gaddum and Kwiatkowski (1938) and was intended to make the rate of outflow independent of the degree of contraction of the vessels of the ear. The "resting" perfusion pressure was usually 10–15 cm. water, but on stimulation of the nerves the pressure rose owing to vasoconstriction.

The perfusing fluid was equilibrated with 5 per cent carbon dioxide in oxygen before perfusion started, and as finally evolved had the composition NaCl 9, KCl 0.21, $CaCl_2$ 0.24, $NaHCO_3$ 0.12, glucose 1 g./litre with ascorbic acid (10^{-5}) and ephedrine hydrochloride (2.5×10^{-6} of base). In a few experiments, the ephedrine was omitted at first and introduced later. The purpose of using half the usual concentration of potassium was to reduce interference in the biological assays; one-quarter the usual bicarbonate was used in order to render the solution somewhat less alkaline (Bülbring, 1944) and so preserve any amines liberated into the vessels. The perfusion was carried out at room temperature.

For stimulation of the nerves, a unipolar electrode was used, the "earth" terminal being a piece of brass gauze wrapped in saline-wetted cotton wool and placed behind the head. Stimulation was by alternating current (50 cycles, 5–10 volts) for 10–20 seconds in every minute for 10–15 minutes. The voltage in the first stimulation period was that which elicited a good contraction when tested on a voluntary muscle, but this was increased in succeeding periods in order to ensure that adequate stimulation was obtained. Continuous stimulation for the whole period of collection of the sample and intermittent stimulation by "square" pulse waves of different durations and frequencies were also tried, but had no advantages.

Procedure.—When the perfusing fluid was clear of blood the collection of samples was commenced. The outflow from the venous cannula was collected in ice-cooled centrifuge tubes, containing solid ascorbic acid (1 mg./10 ml.). Each sample was collected over 10–15 minutes and, in any particular experiment, the samples obtained were of approximately the same volume, since the rate of flow was kept constant by the perfusion device used. As soon as possible after collection the samples were centrifuged (2,000 r.p.m. for 5 min.) to bring down any cells, and the supernatant fluid was transferred to test-tubes and immediately acidified with 2–3 drops of 0.15 N-hydrochloric acid.

After one or more control samples had been taken the electrode was placed under the cleared nerves, or under the nerves and artery if the two had not been separated by dissection. Stimulation was applied intermittently during the sampling. Usually, several "stimulation" samples were collected, interrupted by periods of rest during which control samples were again taken.

Estimation of adrenaline and noradrenaline.—In the first series of experiments these substances were estimated by using several different tests on each sample and calculating the concentrations of the two amines by means of appropriate formulae. The biological methods were those described by Gaddum, Peart, and Vogt (1949) and Gaddum and Lembeck (1949). Shaw's colorimetric test was also used (Shaw, 1938).

In some assays on the rat's uterus and colon the control samples had a stimulant action owing to interfering substances. When these were present the effects of active samples were compared with those of the control samples with added adrenaline (cf. Peart, 1949).

In order to test for the presence of interfering substances which, without being actually stimulating, might have a "masking" effect on small amounts of adrenaline, that amine was added to control samples and this mixture was compared with standard solutions of adrenaline. Down to an adrenaline concentration of 25 mμg./ml. not more than a 20 per cent discrepancy on the rat uterus was found, and usually there was no appreciable difference.

In the second series of experiments a preliminary direct test was carried out on the rat's uterus, and then the two amines were estimated independently after separation by paper chromatography.

Each sample (4–6 ml.) was placed in a 2.5×9 cm. centrifuge tube with a further 2–3 drops of 0.15 N-HCl and 1 mg. ascorbic acid. To each sample 10 ml. ethanol was added. The tubes were left in the refrigerator for 0.5 hour and then centrifuged (2,000 r.p.m. for 15 minutes). The supernatant fluid was transferred to a 50 ml. round-bottomed Quickfit and Quartz flask, the tube being washed with about 2 ml. alcohol which was also transferred. A little whitish residue was sometimes left in the tube. The mixture in the flask was evaporated to dryness *in vacuo* at a temperature not exceeding 60° C. (external temperature) and the flask allowed to cool with the suction still on. The flask was then opened and the inside leached three times with 1, 0.5, and 0.5 ml. alcohol saturated with sodium chloride. Potassium salts are very insoluble in this mixture so that interference in the assays by potassium was minimized (Barsoum and Gaddum, 1935). The leachings from two or more control or stimulation samples were usually pooled by transferring them to another 50 ml. flask. The contents were evaporated *in vacuo* and taken up in 0.75 ml. acid alcohol (0.1 per cent (v/v) conc. HCl in ethanol). This was applied to a cylinder of filter paper for chromatography. The flask was washed with a further 0.2 ml. acid alcohol which was also applied. Attempts were made to keep the total original volumes of the pooled control samples and the pooled stimulation samples approximately equal.

The application of the acid alcohol extracts to the paper, development of the chromatogram with phenol as solvent, elution of the appropriate strips of paper containing adrenaline and noradrenaline, and the assays of these amines on the rat's blood pressure preparation have been described by Crawford and Outschoorn (1951).

The effects of ascorbic acid on the biological tests were investigated. Ascorbic acid (10^{-3}) was used in the tests described by Gaddum, Peart, and Vogt (1949), and did not cause interference. The tests were repeated with five and ten times this amount, the solutions having been acidified with HCl similarly to the samples of the rabbit's ear effluent and made almost neutral with solid sodium bicarbonate before assay on the rat's uterus and colon. The sodium ascorbate did not affect the responses of these tissues. In the chromatographic method, however, much higher concentrations might have occurred in the solutions used in the assay. Standard mixtures of adrenaline and noradrenaline (2 μg./ml. of each) containing ascorbic acid in concentrations of 1.5, 10, and 50 mg./100 ml. were therefore chromatographed and assayed on the rat's blood pressure preparation. The percentage recoveries all agreed closely, irrespective of the amounts of ascorbic acid present originally, and were of the order found by Crawford and Outschoorn (1951). These results indicate that for good recoveries of adrenaline and noradrenaline concentrations of ascorbic acid above 10^{-5} are unnecessary but not disturbing.

The same amounts of adrenaline and noradrenaline were chromatographed with ephedrine hydrochloride (10^{-5} as base) alongside "blank" ephedrine solutions of the

same concentration. Assayed on the rat's blood pressure preparation, the "blanks" showed no difference from equal volumes of saline, while the adrenaline and noradrenaline were recovered to the same extent as without ephedrine. In addition, noradrenaline added to "blank" ephedrine eluates after chromatography showed no "masking" when compared with standard solutions of noradrenaline.

Attempts were made to find out the fate of ephedrine subjected to this method of chromatography and to compare it with that of adrenaline and noradrenaline. About 2 mg. ephedrine in 2×10^{-3} solution was applied on a 12 cm. line alongside a control spot of an adrenaline–noradrenaline mixture. After 22 hours' development the paper was washed in benzene and dried. The ephedrine portion was sprayed with 10 per cent ninhydrin in water, allowed to dry again, and sprayed with 10 per cent sodium carbonate solution. The adrenaline–noradrenaline strip was developed with ferricyanide in the usual way. The ephedrine showed up at the edge of the solvent, having travelled much faster than the adrenaline. For an average solvent flow of 27.5 cm. the mean RF values (taken from the centres of the coloured areas) were: ephedrine 0.85, adrenaline 0.57, and noradrenaline 0.29. The ephedrine was thus well away from the portion of paper which would have contained the adrenaline had this been present in the solution analysed.

The effect of ephedrine on the rat's blood pressure preparation was tested by injecting volumes of up to 0.4 ml. of solutions from 10^{-3} to 10^{-6}. None of the effects was distinguishable from those of saline injections. Dilutions made in the same way, and with the same pipettes, from the same stock solutions of adrenaline and noradrenaline in saline, and in saline containing ephedrine (10^{-5}) were also compared. No differences were observed in the pressor responses. It is generally agreed that low concentrations (10^{-6}–10^{-7}) of ephedrine synergize where high concentrations (10^{-4}) antagonize the effects of adrenaline (Gaddum and Kwiatkowski, 1938; Jang, 1940; West, 1947). Neither effect was noted on the rat's blood pressure over a period of about two hours. It is possible that intermittent injections of ephedrine over a longer period might have made these effects evident, but this was not tested. In any event, as estimates of the amines were made by comparison with standard solutions, it is unlikely that any sensitization by ephedrine would have introduced an error into these estimates.

In the other biological tests, concentrations of ephedrine above 10^{-5} were found to produce definite effects and to potentiate those of standard adrenaline solutions. These effects were most marked on the rat's colon and were less on the rabbit's ear and rat's uterus. Smaller concentrations of ephedrine did not have these effects, and one-fourth the average threshold concentration was selected as suitable for the perfusing fluid. The existence of unusual sensitivity to ephedrine in any of the biological tests would have been brought to light when control samples, containing ephedrine only, were tested. No effects of any significance were found. Ephedrine is known to be without effect on the results of Shaw's chemical test (Gaddum and Kwiatkowski, 1938).

In assays of the final eluates on the rat's blood pressure preparation, injections of samples from some experiments had depressor effects, whereas those from others did not. The results of assays were checked by diluting standard noradrenaline (1 in 10) with eluates of the noradrenaline or adrenaline strips of the control samples collected when the nerves had not been stimulated. The concentrations of these dilutions were made to contain 100–250 mμg. noradrenaline per ml.; if the eluates had depressor properties, these concentrations of noradrenaline were often completely "masked"; if the eluates were pressor, their "masking" effect was either small (20 per cent) or absent.

The results described below were those obtained by actual comparison with standard noradrenaline with no correction for masking. They are therefore often underestimates. Since the occurrence of depressor effects did not appear to be related to the volume of original sample concentrated and chromatographed, and since all the

samples from a particular experiment either did, or did not, prove depressor, the ability to obtain a result of an assay apparently depended on the relative insensitivity of the rat used for the assay to the interfering substances present. It was impracticable to do a preliminary test on one (control) sample and in the event of there being depressor effects prepare another rat in the hope that it would prove more satisfactory.

RESULTS

In some experiments the activity of the effluent was too low to be detected by direct tests, and in others stimulation of the nerve had no apparent effect, but in eight experiments there was clear evidence by at least two methods that the samples after stimulation were more active than control samples. This result was obtained not only by individual assays of the samples against standard solutions but also by direct comparison of the control and stimulation samples in the same biological tests. The results obtained by each of the tests used are given in Table I. By this direct comparison and assay, increased activity due to stimulation was shown by the rat's uterus 9/10 times, by the rat's colon 3/5 times, by the rabbit's ear 2/2 times, and by Shaw's test 5/5 times. Table II shows the results of tests on the active samples collected during and after stimulation when the increase due to stimulation was shown by at least two tests.

TABLE I

RESULTS OF ASSAYS OF SAMPLES BY DIFFERENT TESTS

Test	Exp. No.	Dose ratio Nor./Adr. for equal responses	Adrenaline equivalents (mμg./ml.)		
			Control	During stimulation	After stimulation
Rat's uterus	4	100	<2	2.5	1.8
	5	15	1	1	7.9
	6	150	1.87	5.8	3.3
	9	150	1.8	3.7	5
	11	100	<2.5	<1.25	<1.25
	12	100	1.8	3.7	<1.25
	13	75	<1.25	60	—
	16	100	3.7	15	—
	17	150	<5	5	5
	19	75	<2.5	3.7	—
Rat's colon	4	1	<125	750	375
	5	1	187	187	375
	6	0.75	<125	<125	<125
	13	1.1	—	750	—
	16	1.5	—	33	—
	17	2	<250	<50	82
	19	0.5	<300	<300	—
Rabbit's ear	5	3	—	—	10
	6	1.5	4	8	7.5
	13	0.75	—	3,000	—
	16	3.7	7.5	15	—
Shaw's test	9	13.5	9	106	17
	11		26	25	70
	12		15	30	—
	13		20	112	—
	16		38	110	—

TABLE II

RESULTS OF TESTS ON SAMPLES COLLECTED DURING AND AFTER STIMULATION

Exp. No.	Adrenaline equivalents (mμg./ml.)				Concentrations calculated from uterus and colon results (mμg./ml.)		Percentage content of adrenaline
	Shaw's test	Uterus	Colon	Ear	Adrenaline	Noradrenaline	
4	—	2.5	750	—	(−5)	747	0
	—	1.8	375	—	(−1.9)	373	
5	—	7.9	375	10	(−17.1)	367	0
6	—	5.8	<125	8	—	—	
	—	3.3	<125	7.5	—	—	
9	106	3.7	—	—	—	—	
	17	5	—	—	—	—	
12	30	3.7	—	—	—	—	
13	112	60	750	3,000	50	759	6.2
16	110	15	33	15	14.5	28	34
17	—	5	82	—	4.5	72	6

The estimates of the adrenaline equivalent with the uterus were nearly always much lower than those given by Shaw's test or by the colon or the ear. This fact itself shows that adrenaline was not the only substance present, and could be explained by the presence of noradrenaline. The equivalent concentrations of these two substances were calculated from the results obtained with the uterus and colon by the formulae used by Gaddum and Lembeck (1949) and others.

In Table II the concentrations of adrenaline and noradrenaline calculated in this way show in some instances " negative " values for adrenaline (experiments 4 and 5). These minus values are a measure of the error of the assay methods when a small amount of adrenaline is present in a mixture with a relatively larger amount of noradrenaline, i.e., when the percentage content of adrenaline is below ten. Gaddum and Lembeck (1949) have shown that in such assays only the calculated noradrenaline figure can be accepted as reasonably accurate. The replacement of the minus values by zero (i.e., no detectable adrenaline) is considered unjustifiable. It is doubtful whether all the results can be explained by the presence of these two amines, but the results do not justify speculation about the possible presence of other substances.

The results in Table III provide better evidence for the conclusion that adrenaline and noradrenaline are both released when the nerve is stimulated. In these experiments, noradrenaline was estimated on the rat's blood pressure after chromatographic separation from adrenaline and impurities. It was not possible to estimate adrenaline in the same way, since the rat's blood pressure is not sensitive enough to detect these very small amounts of adrenaline, and the rat's uterus is too sensitive to interfering substances coming from the paper itself. The estimates of adrenaline given in Table III were therefore obtained by testing samples taken from the unconcentrated extracts before chromatography. The amounts of noradrenaline found would not affect the results of those tests, since the rat's uterus was never less than 100 times as sensitive to adrenaline as it was to noradrenaline. The evidence in

TABLE III

Minute output of adrenaline and noradrenaline, calculated from results of estimates in pooled samples of effluent. Adrenaline estimated directly with the rat's uterus; noradrenaline estimated with the rat's blood pressure after chromatographic separation.

Exp. No.	Control samples		Samples collected during stimulation		
	Adrenaline (mμg.)	Noradrenaline (mμg.)	Adrenaline (mμg.)	Noradrenaline (mμg.)	% Adrenaline content
25	<0.33	Depressor	0.60	Depressor	—
27	0.12	<4.17	0.34	<6.25	<2.5
29	0.68	<8.33	1.00	12.50	7.4
30	<1.41	Depressor	1.45	Depressor	—
31	<0.81	<3.33	0.82	1.43	36.5
32	<0.45	<3.38	0.71	3.75	15.9
34	0.84	<2.07	0.60	<2.07	>22.5
35	0.42	<1.67	0.48	3.12	13.3
36	<0.52	<8.33	0.30	<3.12	>8.8
39	<0.43	<0.83	0.59	1.10	34.9

Tables II and III that the substance measured by these tests on the rat's uterus was adrenaline was thus not very good ; it was certainly not noradrenaline or any other substance which is known to occur in the body. If it is true that these results give an estimate of adrenaline, then this substance was present in all the samples collected during stimulation. The amounts present in control samples were generally too small to be detected, and in 8 of the 10 experiments (Table III) there was evidence that adrenaline was present after stimulation in higher concentration than before.

Noradrenaline was detected in 5 of the 10 samples (Table III) collected during stimulation, and in each of these samples the concentration was greater than in the corresponding control samples. The identification of this substance as noradrenaline depends upon the fact that it moved up the paper at the same rate as noradrenaline.

These results show that both amines were present in increased amounts after stimulation. The percentage content of adrenaline in the samples collected during stimulation varied between 7.4 and 36.5 per cent.

Discussion

The results recorded here show that both adrenaline and noradrenaline are released during stimulation of the nerves to the rabbit's ear. The percentage of adrenaline was often much higher than that found for other nerves, so that it is perhaps not surprising that Gaddum and his co-workers failed to detect the simultaneous liberation of noradrenaline. The concentrations of both amines were very low and it was difficult to get satisfactory results even with the improved methods now available. The results of the direct tests shown in Tables I and II prove that adrenaline was not the only substance present, since the test on the uterus gave lower estimates than the other tests. This test has a higher dose-ratio (noradrenaline/adrenaline) than any other known test and is therefore particularly useful in experiments of this type. Gaddum and Kwiatkowski (1939) used the hen's rectal caecum, which also has a fairly high dose-ratio, and did find that the adrenaline equivalent in this test was sometimes lower than that on the frog's heart. This might have

suggested the presence of noradrenaline, but the evidence was not convincing and was neglected. The specific test described by Shaw (1938) proved that adrenaline was present and the facts did not justify the theory that the two amines were both present.

In the present experiments, only Shaw's ordinary test was applied, and although the results are similar to Gaddum and Kwiatkowski's the discrepancy found between the adrenaline equivalent obtained by it and with the rat's uterus indicates that in the chemical method some other reactor with the colour reagent was also being estimated, since the effluent did not contain any substances which " masked " the effect of adrenaline added to control samples and assayed on the rat's uterus.

As previously shown by Gaddum and Kwiatkowski (1938), the addition of ephedrine to the perfusing fluid was essential in order to obtain a high yield of sympathin. The reason they assumed for this phenomenon was the inhibition of amine oxidase by ephedrine. Recent evidence in favour of their hypothesis was obtained when Thompson and Tickner (1951) were able to demonstrate the presence of the enzyme in rabbit ear vessels. It was further shown by Burn and Robinson (1951) that the potentiation of the effects of injected amines was lost when the enzyme had apparently disappeared after prolonged perfusion. The results in Table IV show the effect of adding ephedrine to the perfusing fluid.

TABLE IV

RESULTS OF ASSAYS ON THE RAT'S UTERUS

Plain Locke 1.15 p.m.			Ephedrine-Locke 3.30 p.m.		
Sample	Time p.m.	Adrenaline equivalent (mμg./ml.)	Sample	Time p.m.	Adrenaline equivalent (mμg./ml.)
Control	2.15	<1.25	Control	4.00	<1.25
Stim.	2.30	<1.25	Stim.	4.15	1.25
Stim.	3.00	<1.25	Stim.	4.50	1.87
			Stim.	5.05	1.25

The low concentrations obtained show that it would not have been possible, even now, to prove that the two amines were both present without the use of concentrated extracts and chromatography. The evidence still does not exclude the possibility that other active substances besides adrenaline and noradrenaline are released.

SUMMARY

1. The sympathetic nerves were stimulated in rabbit ears perfused with salt solutions and the effluent tested for sympathomimetic amines by colorimetric and biological methods, aided by chromatography.

2. The release of adrenaline has been confirmed, but evidence is presented that it is accompanied by larger amounts of noradrenaline.

3. The percentage adrenaline content was generally higher than that usually found after stimulation of other adrenergic nerves, but there was no evidence of any qualitative differences between sympathetic nerves to the rabbit's ear and those to other tissues.

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THE PROTECTIVE ACTION OF ANTIHISTAMINES IN THE ANAPHYLACTIC MICROSHOCK OF THE GUINEA-PIG

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It has been known for some time that the antihistamines protect against anaphylactic shock. Bovet, Horclois, and Walthert (1944) have shown this for mepyramine (neoantergan, Anthisan), and, subsequently, it has been shown for many other antihistamines. These authors observed protection from the fatal outcome of shock. An exact threshold dosage for this protection could not be determined, because the amounts of antigen and antibody which react together and thus lead to fatal shock are variable. An exact quantitative comparison of the various antihistamines is therefore, as Feinberg *et al.* (1950a) have pointed out, not possible. Feinberg *et al.* (1950b) attempted an approximate comparison by contrasting the number of deaths in anaphylactic shock with the number of protected guinea-pigs. We have selected from their material the more familiar antihistamines and reproduced their results in Table I, from which it will be seen that there are certain differences: promethazine, chlorcyclizine, chlortrimeton, and, perhaps, mepyramine seem to have a strong protective action. This group is followed by tripelennamine and diphenhydramine, whose action is weaker ; antazoline is the weakest. These differences, however, are uncertain, as only one or two doses of most drugs were tried. Within the groups, differentiation is impossible.

We used the microshock method (Herxheimer, 1952a) in order to obtain a quantitative comparison. A mild repeatable shock of constant strength was applied by inhalation of the aerosolized antigen. The fatal outcome was prevented by removing the animal from the aerosol just before convulsions would have occurred. The time required to reach this point is called the preconvulsion time. After one exposure re-formation of antibodies takes place which are removed by the next exposure. The longer the interval between two exposures the more antibodies are re-formed. By varying the interval for each animal we could determine the period required for the formation of sufficient antibody to produce a given preconvulsion time on re-

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TABLE I

DEATH RATE OF GUINEA-PIGS IN ANAPHYLACTIC SHOCK UNDER THE PROTECTIVE INFLUENCE OF ANTIHISTAMINES (AFTER FEINBERG *et al.*, 1950B)

The first figure indicates the number of deaths, the second figure the total number of animals used

Substance	Dosage			
	0.1	1.0	3.0	5.0 mg./kg.
Diphenhydramine	6/10	13/20	3/10	1/8
Tripelennamine		10/20	3/10	
Mepyramine				
Antazoline		10/10		
Promethazine			0/5	
Chlortrimeton			1/5	
Chlorcyclizine		3/10	0/5	

exposure. If this period is adopted as the interval between exposures, the preconvulsion time will remain approximately constant. Its increase under drug influence therefore signifies a protective effect.

METHOD

We used groups of 4–9 guinea-pigs for each single experiment. The animals were sensitized by intramuscular injection of 0.7–0.8 ml. of a 5 per cent solution of crystalline egg albumin. After 21 days the animals were exposed to aerosolized antigen, and the preconvulsion time was determined. At the first exposure half the usual antigen concentration was used, because the preconvulsion time may be very short (less than 40 sec.) and the first signs of dyspnoea may be followed rapidly by convulsions and death. Further exposures were carried out at intervals of 2–3 hours or 24 hours. These led to a partial desensitization and thus to an increase in preconvulsion time. When the preconvulsion time had reached 80–120 seconds, the next exposure was carried out after 3–4 days. In some animals it then remained fairly constant, but in others it increased further. For the latter animals the interval was increased to 6–7 days, and usually this proved to be the correct period for maintaining a constant preconvulsion time. When exposures with the appropriate intervals had been repeated, groups of animals with the same intervals were formed and the drug experiments started. Each exposure under the influence of a drug was followed by a control exposure without it. The mean of the preconvulsion times before and after drug exposure was contrasted with the preconvulsion time under drug influence. The drawbacks of this method are, as pointed out elsewhere (Herxheimer, 1952a), the individual variability of guinea-pigs and the difficulty of assessing the convulsion point accurately. Some animals showed great variations between two preconvulsion times, varying sometimes from 90 to as long as 170 seconds, or vice versa. Such extreme fluctuations in the control times, although rare, increase the inherent variability in results from different animals and necessitate a large number of experimental groups.

We examined not only the influence of a number of antihistamines, but also that of atropine sulphate, procaine, and procaine amide. The last two were given intraperitoneally 20 min. before exposure, the others intramuscularly 60–90 min. before exposure. The latter period was chosen because Bain (1949) found that the maximum effect of various antihistamines was reached 2–3 hours after oral administration. Clinically, a pronounced drug effect is observed with most antihistamines one hour, and with Promethazine 1.5 hours, after they have been taken by mouth. As intramuscular injection must be

expected to reach its maximum effect after a much shorter time, we felt that exposure 60–90 min. after injection would show the drug effect near its maximum.

In three experiments not included in the results reported in this paper, we gave antihistamines (promethazine, 0.5 and 2.0 mg./kg.; diphenhydramine, 3.0 mg./kg.) intraperitoneally 45–60 min. before exposure. The results differed little from those produced by intramuscular injection. We are aware of the fact that protection can also be achieved by administration of the drug in the form of an aerosol. Our results with this method will be reported separately

In three other experiments not included in the results reported in this paper we used animals sensitized by injection to horsehair and dander extract or to castor-bean extract (Bencards), and shocked by the aerosol of the same extract. The degree of protection achieved against these antigens was of the same order as in our other experiments, but the number of experiments is too small to permit a comparison.

RESULTS

The results and their statistical analysis are shown in Tables II–VI and Fig. 1. The method used for measuring the protective effect of a single dose of a drug is illustrated in Table II, which shows the results for the group of six guinea-pigs

TABLE II

EXAMPLE OF THE METHOD OF CALCULATION OF PERCENTAGE PROTECTION

The protection given by 12 mg./kg. of chlorcyclizine

(1) Animal	(2) Control preconvulsion time (mean of 2 controls) in sec. C	(3) Drug preconvulsion time in sec. T	(4) Percentage protection $100\,(1-C/T)$
1	140	480	71
2	117	450	74
3	74	180	59
4	113	240	53
5	92	230	60
6	118	180	34

Mean protection: 58.5%. Standard deviation: 14.3. Standard error of mean: ±5.9

treated with the highest dose (12 mg./kg.) of chlorcyclizine. Column (2) shows the mean, C, of the preconvulsion times before and after treatment, and column (3) shows the preconvulsion time, T, under the influence of the drug. As a measure of " percentage protection " we have calculated, in column (4), the quantity $100\,(1-C/T)$. If the control and treatment preconvulsion times, C and T, are equal, the percentage protection is zero ; if the drug gives complete protection, T is effectively infinite, and the percentage protection is 100. Occasionally, with individual animals at low doses, T is less than C, and the percentage protection is negative.

Table III shows, for each dose, the mean of the individual values of percentage protection, and the standard error of the mean. For each drug the mean responses tend to increase with increasing dose. The exact nature of the relationship between percentage protection and dose is rather speculative, but in view of the upper limit of 100 per cent to the scale of protection, and the likely lower limit of zero at small doses, some sort of sigmoid relationship might be expected. The results for some

TABLE III

MEAN PERCENTAGE OF PROTECTION AND STANDARD ERROR FOR EACH DOSE

	Dose mg./kg.	No. of animals	Mean percentage protection ± standard error	Estimated mean from fitted curve
Antazoline (antistine) ..	0.2	9	−20.9±17.2	8
	0.3	5	5.4± 9.3	17
	0.5	5	47.2± 3.6	40
	1.0	6	42.5± 6.6	58
	3.0	7	73.9± 3.9	67
	3.0	5	61.6± 7.9	67
	12.0	4	52.8± 2.3	57
	20.0	6	39.7± 6.1	40
Atropine	0.11	7	1.4±12.7	1.3
	0.22	8	18.0± 9.3	6.8
	0.325	7	14.7± 5.1	19.0
	0.65	6	60.2± 6.8	49.2
	1.3	6	43.8± 9.1	58.7
	2.6	7	55.0± 8.2	59.9
	6.35	4	45.5±15.8	60.0
Chlorcyclizine (histantin) ..	0.025	6	−4.7±10.2	5.6
	0.05	5	2.0±18.7	10.7
	0.08	8	−19.0±16.4	15.8
	0.2	7	29.9± 2.8	29.4
	1.0	6	48.5±10.4	50.8
	6.0	5	58.2± 2.4	58.5
	12.0	6	58.5± 5.9	59.3
Chlortrimeton	0.01	6	−6.5±23.5	11.0
	0.05	4	22.0±10.2	21.8
	1.0	6	58.7± 9.4	55.8
	2.8	5	67.4± 3.9	68.1
Diphenhydramine (benadryl)	0.025	5	−7.8±10.3	2.6
	0.05	6	−18.7±10.3	5.5
	0.2	6	31.0±10.2	13.6
	0.9	7	33.3± 3.2	31.4
	2.0	6	17.3±11.5	44.9
	6.0	6	44.7± 9.7	64.2
	12.0	5	80.8± 4.0	74.6
	12.0	5	73.2± 3.4	74.6
Mepyramine (neoantergan, anthisan)	0.01	5	14.6± 3.5	6.3
	0.025	7	5.7±11.9	12.9
	0.05	7	25.7± 6.6	20.8
	0.1	6	32.8± 7.7	31.0
	0.167	6	22.5± 8.0	39.3
	0.4	6	59.7± 5.5	52.9
	1.0	7	64.0± 6.3	63.2
	3.0	7	78.6± 5.7	70.1
	6.0	5	57.6± 7.2	72.3
Promethazine (phenergan) ..	0.01	5	−10.4±10.8	0.4
	0.025	6	22.0± 6.8	4.1
	0.05	9	1.7±15.7	18.4
	0.1	8	46.3± 3.3	47.2
	0.1	9	44.0± 6.8	47.2
	0.25	7	78.9± 5.3	66.9
	0.5	7	72.6±10.8	69.4
	0.75	6	68.8± 7.4	69.8
	0.75	7	75.6± 5.1	69.8
	1.5	7	66.0± 6.7	70.0
	3.0	7	76.6± 7.0	70.0
Tripelennamine (pyribenzamine)	0.01	6	50.5± 5.2	42.6
	0.01	4	27.8± 9.1	42.6
	0.02	6	24.2±11.3	45.4
	0.05	6	54.0± 6.3	49.1
	0.25	5	42.2±11.9	55.7
	1.0	5	62.2± 8.2	61.1
	3.0	5	54.2± 8.4	65.3
	30.0*	6	87.2± 9.3	73.3

* This dose caused violent convulsions lasting about 20 minutes. Exposure to antigen aerosol was carried out 40 minutes later.

drugs suggest an upper limit at less than 100 per cent. We have therefore followed the procedure of guessing an upper limit to the percentage protection for each drug, and fitting a logistic curve relating percentage protection to log-dose. The equation of the curve may be written

$$Y=\frac{L}{1+e^{-b\,(x-x_0)}}$$

where Y is the mean percentage protection expected at a dose whose logarithm is x units, L is the assumed upper limit to Y, and the two constants b and x_0 are estimated from the data. Of these, b represents the steepness of the increase of Y with x, and x_0 is the logarithm of the dose required to give a protection of 0.5 L per cent. More precisely, the maximum gradient of the curve, which occurs at a response of 0.5 L per cent, is measured by $b'=0.25Lb$; b' is a convenient measure of the relative steepness or " slope " of the different curves.

This procedure was not suitable for antazoline, for which the responses appear to rise to a maximum at a dose of about 3 mg./kg. and then decrease at higher doses. For this drug a freehand curve has been fitted to the results.

The method used for fitting the logistic curve is similar to that suggested by Emmens (1940, pp. 221–3). A more precise method (that of maximum likelihood) has been described by Finney (1952, Section 47 and Appendix II), in which the upper limit L is estimated from the data ; the present data are too variable, and the suitability of the logistic curve too questionable, to justify undue refinement. The assumed values of L, and the estimated values of the " slope," b', and of x, are shown in Table IIIa. The high values of χ^2 given in Table IIIa show that the fit of some of the curves is bad in the strict statistical sense. The total value of χ^2 is 87.0 on 40 degrees of freedom ($P<0.001$) : the observed mean responses diverge from the fitted curves to a greater degree than would be expected by chance. This divergence may occasionally be due to the use of an inadequate model ; for some drugs the response curve may reach an upper limit more suddenly than does the logistic, or, like that

TABLE IIIa

ESTIMATED CONSTANTS FOR EACH LOGISTIC CURVE, WITH MEASURE OF GOODNESS OF FIT

Drug	Assumed upper limit L for % protection	Estimated " slope" b' ± standard error	Estimated log-dose x_0 giving protection of $\frac{1}{2}L\%$ ± standard error	χ^2 index for goodness of fit, with No. of degrees of freedom	Significance of χ^2
Antazoline	—		(Curve fitted by eye)		
Atropine	60	113.7±39.8	−0.39±0.06	8.7 on 5 d.f.	$0.1<P<0.2$
Chlorcyclizine	60	37.2±16.9	−0.69±0.11	5.8 on 5 d.f.	$0.3<P<0.5$
Chlortrimeton	100	29.1±11.8	−0.20±0.27	0.1 on 2 d.f.	$0.90<P<0.95$
Diphenhydramine	100	41.2± 6.2	0.42±0.09	22.1 on 6 d.f.	$0.001<P<0.01$
Mepyramine	75	38.1±12.2	−0.83±0.15	18.8 on 7 d.f.	$0.001<P<0.01$
Promethazine	70	102.5±30.1	−1.12±0.05	17.1 on 9 d.f.	$0.02<P<0.05$
Tripelennamine	100	9.4± 4.4	−1.20±0.47	14.4 on 6 d.f.	$0.02<P<0.05$
			Total	87.0 on 40 d.f.	

for antazoline, may even fall slightly with increases of the dose beyond some optimum level. Usually, however, there is no *systematic* divergence from the logistic curve, and the discrepancies are probably due to heterogeneity between the groups of animals allotted to different doses. The standard errors of the estimated quantities given in Table IIIa take into account this heterogeneity; the heterogeneity factor, $87.0/40 = 2.175$, has been used for each drug (Finney, 1952). The estimated mean response at each dose is shown, for comparison with the observed value, in Table III; the fitted curves are also plotted in Fig. 1.

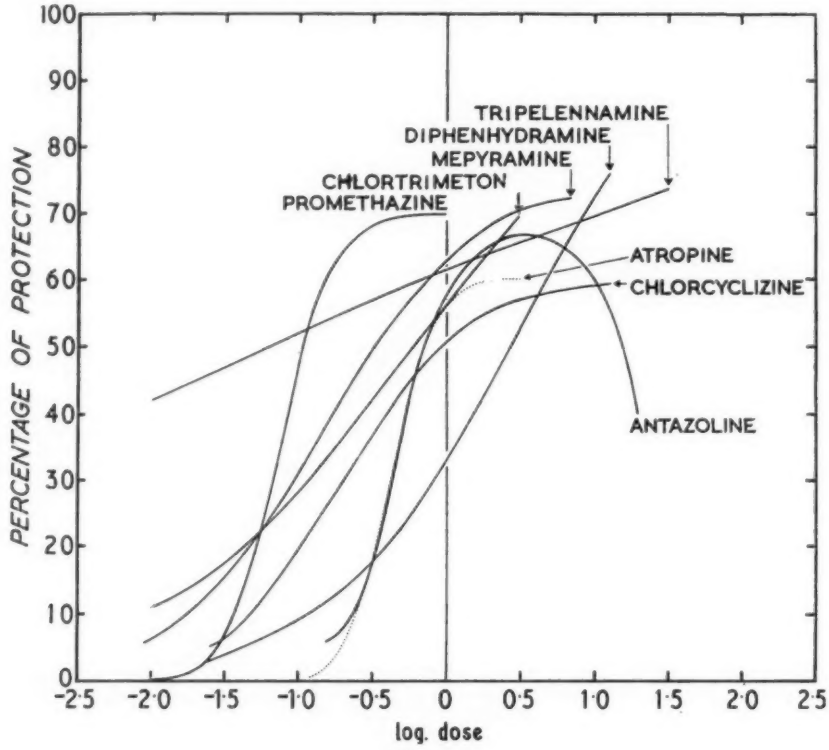

FIG. 1

Some protection against anaphylactic shock (expressed as increase of preconvulsion time) was given by all the antihistamines which we tried. This protection, however, was never complete except at very high dosages, which would be toxic for man, and even then it was present in some animals only. The degree of protection depends on the dosage. With all but one substance (tripelennamine) the smallest doses used gave little or no protection (Fig. 1). Table IV shows the lowest doses at which the mean protection was significant. This dosage differs from one substance to another, and is lower than the therapeutic dosage used in man. As the dose is increased, protection rapidly becomes more pronounced, but for most substances it seems to approach a maximum value below 100 per cent. For each substance the dose beyond which there is no significant increase in protection is shown in Table V. Again, there are considerable differences between substances, but, except for atropine, these doses are of a similar order to the therapeutic doses

TABLE IV

LOWEST DOSE AT WHICH SIGNIFICANT PROTECTION OCCURS

(Chlortrimeton has been omitted from this Table, as no intermediate doses between 0.05 and 1 mg./kg. have been tried)

	mg./kg.
Antazoline	0.5
Atropine	0.325
Diphenhydramine	0.2
Chlorcyclizine	0.2
Promethazine	0.025
Mepyramine	0.005(?)*
Tripelennamine	0.005*

* See addendum.

TABLE V

LOWEST DOSE BEYOND WHICH NO SIGNIFICANT INCREASE IN PROTECTION OCCURS

(Chlortrimeton has been omitted from this Table, as no intermediate doses between 0.05 and 1 mg./kg. have been tried)

	mg./kg.
Antazoline	3.0*
Chlorcyclizine	1.0
Atropine	0.65
Mepyramine	0.4
Diphenhydramine	†
Promethazine	0.25
Tripelennamine	‡

* Higher doses lead to a decrease of protection.
† Observed protection remains fairly constant between 0.2 and 6.0 mg./kg. with a significant rise at the excessive dose of 12.0 mg./kg.
‡ Observed protection remains fairly constant between 0.01 and 3.0 mg./kg., with a significant rise at the excessive dose of 30.0 mg./kg.

used in man. Thus, doses far in excess of those used in man usually failed to give complete protection. With antazoline, the excessive dose of 20 mg./kg. gave less protection than 3 mg./kg. and 12 mg./kg.

Atropine, which has a weak antihistaminic action, ranges with the weaker group of substances tried. Protective effect in anaphylactic microshock exists at 0.325 mg./kg. and starts possibly at 0.22 mg./kg., doses which would be toxic in man. Procaine also has a protective effect, but it is weaker than any of the other substances. Whether procaine amide has a protective action is uncertain (Table VI).

TABLE VI

PROTECTIVE EFFECT OF PROCAINE AND PROCAINE AMIDE

Drug	Dose mg./kg.	No. of animals	Mean % protection ± standard error	Standard deviation
Procaine	3	5	−12.6±10.5	23.6
	6	5	35.6±14.7	32.8
	10	3	32.0±15.2	33.9
	25	5	36.2±11.7	26.1
Procaine amide	3	5	15.0±14.5	32.4
	10	3	45.0± 1.7	3.0
	25	5	6.4±15.6	34.8
	50	5	15.6±24.7	55.3

The individual reaction of the animals to the same drug varied considerably. As a rule, the animals which were strongly protected by one antihistamine were also protected by the others. There were, however, exceptions to this rule: Animal 180, for instance, was well protected by medium doses of tripelennamine and chlortrimeton, but not by a large dose (3.0 mg.) of promethazine. Animal 158 was well protected by 0.01 mg. tripelennamine, but not by 2.0 mg. diphenhydramine. Animal 174 was little influenced by 0.25 mg. tripelennamine, but well protected by 0.1 mg. promethazine. Animal 186 was not protected by 0.25 mg. tripelennamine or 0.2 mg.

diphenhydramine, but well protected by 0.5 mg. of the much weaker antazoline. We noticed these differences only when we had completed most of our experiments. As the total number of animals used was large, and as animals were changed haphazardly for each experiment, it was rare that the same animal was used with corresponding doses of different antihistamines. If the experiments had been arranged with a view to observing individual differences, we might have seen more such examples. This difference in individual responses is interesting, as it occurs also in man.

Discussion

Our results seem to be in approximate agreement with those of Feinberg *et al.* (1950b). Some antihistamines (promethazine, mepyramine, and chlortrimeton) have, in non-toxic doses, a much stronger action than others. The weaker group, in our experiments, included tripelennamine, diphenhydramine, chlorcyclizine, and antazoline. Chlorcyclizine was included by Feinberg *et al.* in their stronger group. Apart from these gross differences in strength, each substance appears to possess some characteristic behaviour by which it differs from all others. Tripelennamine, for instance, protects in smaller amounts than the others, but the protection afforded by a medium dose is unexpectedly weak, like that of chlorcyclizine. At an extreme range, however, which is toxic even for guinea-pigs, it becomes efficient. Diphenhydramine gives a very weak protection even in high dosage, but at an extreme range its protection becomes efficient. The protection by chlorcyclizine is somewhat better, but it does not improve at extreme range. Naturally, these differences can only be detected by experiments with graded dosage. They show that these substances not only have a protective action, but that they differ from each other in characteristic details. These can be best seen from Fig. 1. They can be summarized as follows: The highest degree of protection is given, in doses tolerable for man, by promethazine, mepyramine, and (to a slightly lesser degree) chlortrimeton. This effect is achieved by promethazine with a smaller amount than by mepyramine and by mepyramine with a smaller amount than by chlortrimeton. Tripelennamine and diphenhydramine achieve the same high protection but only with amounts toxic for man. The other substances do not give such a high degree of protection whatever their dose, and for this lesser degree of protection greater amounts are required.

We shall now consider how far anaphylactic microshocks can be compared to attacks of asthma in man. Many authors agree that there is no principal difference between anaphylactic shock in the animal and allergic disease in man. We share this opinion, especially in view of the work of Schild *et al.* (1951), who found that isolated human bronchi of an asthmatic patient reacted in exactly the same way as organs of sensitized animals. The microshocks in our experiments were reactions due to antigen introduced into the respiratory passages of a sensitized animal. The allergic asthma attack in man is caused in the same way. The only, and perhaps important, difference is that in the animal sensitization has been carried out by injection, whereas in man sensitization occurs because there is a congenital tendency (" Anlage ") which is absent in the guinea-pig. In view of this difference we cannot regard both phenomena as exactly the same, but they are closely related. We have been able to elicit the same microshock also when the sensitization was carried out by inhalation instead of by injection. The antigen used was extract of horsehair and dander, and sensitization was not achieved with the same regularity as by

injection. The similarity of both phenomena is evident also from their external appearance. The increasing dyspnoea, the standing of the animals on their front feet to support inspiration, the gasping for air, and the cyanosis are so similar to the acute asthmatic attack that the animal assumes an almost human expression. The onset and course of the dyspnoea have clearly the same mechanism. This statement is supported by the anatomical findings if the animal dies: there is always gross emphysema and also some oedema of the mucous membrane even if the attack is the first of this kind experienced by the animal (Herxheimer and Pagel, unpublished experiments). The degree of oedema is not sufficient to cause fatal emphysema ; bronchial muscular spasm must be the main cause. These findings are very similar to those in human asthma ; there the picture is often complicated by chronic bronchitis, but in patients in whom an acute attack of unusual violence has caused death during an otherwise asthma-free interval (for instance, after a wasp sting or an intracutaneous skin test), the same picture of almost pure emphysema can be seen. These many similarities between the microshock in the guinea-pig and the asthma attack in man justify, in our opinion, their comparison under the influence of drugs.

Such a comparison reveals many parallels. The most striking parallel is the limitation of the protective effect. The antihistamines protect against mild asthmatic attacks, especially against nocturnal attacks in chronic asthma. They are useless in acute or severe attacks, which they neither prevent nor influence (Herxheimer, 1952b). In induced asthma mild attacks can be prevented (Herxheimer, 1949), but moderate or severe attacks cannot. If in a patient an anti-asthmatic effect has been achieved with, say, 25 mg. promethazine, and the asthmatic obstruction becomes more severe, the dose can be successfully increased to 50 or 100 mg. If the obstruction becomes more severe still, a further increase of the dose usually does not give an additional benefit. In all these examples there is a beneficial effect, but it is limited as in the guinea-pig, and an increase in dosage beyond a certain limit is of no value. In both man and guinea-pig there is protection, but a partial protection only.

Another parallel is found in the great individual differences in the drug action. In man we find a number of patients who respond well to several antihistamines, but there are others who respond only to one and not to others. The guinea-pig shows a similar behaviour. Individual animals may occasionally respond very little, if at all, to some particular antihistamine. This variability can also be seen in their response to low dosage. A high dose of an antihistamine usually protects all animals of one group, though at a varying degree. If this dose is decreased to such a level that the mean protection is just significant, it will be seen that some of the individual animals in this group are not at all protected. This is a regular occurrence at such borderline doses.

Further similarities emerge if dosage and order of efficiency are compared. The smallest dosage which affords very pronounced protection in the guinea-pig is usually smaller than that used clinically in man, often one-half to one-third as large, but in man the drug is given orally. As the drugs were given intramuscularly to our animals, the difference does not seem considerable. The order of efficiency, weight for weight, is difficult to assess clinically ; it can be found approximately in those patients who respond well to a number of antihistamines. From such comparisons we have found that promethazine is about 6–7 times stronger than mepyramine.

Diphenhydramine and chlorcyclizine are much weaker, and antazoline is practically useless in asthma. With chlortrimeton and tripelennamine we have not sufficient clinical experience, but it seems to us that the former is almost as strong as promethazine, and the latter stronger than diphenhydramine and chlorcyclizine. Bain (1950) also found similar differences between some of these drugs, although he tested them against skin responses to histamine. In our animal experiments a similar order emerges to that found clinically. A comparison with the procaine action has the same result. In the guinea-pig its action is weaker than that of the weakest antihistamine. In man an anti-asthmatic effect can be observed only after intravenous administration ; it is weak and fleeting. There is some considerable difference between the effect of atropine in the guinea-pig and in man. In human asthma its action is almost negligible. The reason for this difference is probably that the equivalent of the effective dose in the guinea-pig would have toxic effects in man.

The similarity of the drug action in guinea-pig and man throws further light on our views on antihistamine treatment of human asthma. The varying and erratic results of the treatment of sometimes quite unsuitable cases have led some observers to regard this therapy as useless or, at least, as disappointing. This view has tempted some of them to explain some undeniable successes as due to suggestion or to the sedative effect of the antihistamines. One of us (Herxheimer, 1949, 1952b) has given reasons why neither of these explanations could be valid. Our present experiments confirm this view: Suggestions could not have increased the preconvulsion time in guinea-pigs, nor could sedation alone have had this effect. We have given 0.1 mg./kg. of pentobarbitone to a group of guinea-pigs and have not found any change in the preconvulsion time, although the animals showed signs of being tired: they were unsteady on their feet and, at times, closed their eyes. Similar symptoms were noted also after antihistamines, but only after large doses (3 mg./kg.). The animal experiments thus confirm the conclusions drawn from clinical experience.

Our experiments do not show how the antihistamines achieve their protective action. They may counteract oedema of the mucous membrane or muscular spasm, or both. Their action in allergic rhinitis makes it probable that they reduce oedema. The experiments of Schild *et al.* (1951), and our present experiments in which oedema cannot have played a dominant part, make it probable that they also counteract muscular spasm to some degree.

Summary

1. Antihistamines have a protective effect in the mild anaphylactic shock of the guinea-pig.

2. This effect begins with doses in the region from 0.01 0.5 mg./kg. and approaches its maximum with doses from 0.25–3.0 mg./kg. The protective effect consists in delaying the onset of the asthmatic dyspnoea due to the shock. It does not prevent the onset of dyspnoea except in a few animals in which protection is complete.

3. There are considerable differences in the protective strength of the antihistamines tested. There is a stronger group (promethazine, mepyramine, and chlortrimeton) affording a higher degree of protection, and a weaker group (tripelennamine, diphenhydramine, chlorcyclizine, and antazoline). Each of these substances has characteristics of its own.

4. The anti-anaphylactic action of atropine is similar to that of the weaker group. The protective action of procaine is still smaller.

5. Parallels exist between the action of the antihistamines in the guinea-pig and in man. In both, moderate doses give a moderate protection which is improved with an increase in dosage until a limit is reached. In both, there are considerable variations of the effect in the individual animal or patient. In both, the classification of the drugs in groups according to their strength is about the same.

6. As sedative and psychological effects of the antihistamines cannot account for their action in the guinea-pig, it is unlikely that these factors are the reason for their beneficial action in man.

ADDENDUM

Very recently, we have had the opportunity of investigating the protective effect of smaller amounts of tripelennamine and mepyramine than were previously used. We have also examined a wide range of dosage of a new antihistamine, provisionally named 295/C/51 by Burroughs, Wellcome & Co. These results are shown in Table VII. The experiments with tripelennamine confirm our expectation that some protection is conferred by a dose of 0.005 mg./kg. With mepyramine we found a higher level of protection than before with all three doses used. We cannot explain this difference, but as these experiments were carried out three months later than the

TABLE VII

Drug	Dose mg./kg.	Log dose	No. of animals	Mean percentage protection ± standard error
Mepyramine	0.001	−3.00	7	−17.57 ± 9.22
	0.005	−2.30	6	39.17 ± 6.06
			6	17.67 ± 6.64
	0.01	−2.00	6	62.67 ± 5.92
			7	65.71 ± 3.09
	1.0	0	6	80.67 ± 2.82
Tripelennamine	0.001	−3.00	6	−31.83 ± 24.50
	0.005	−2.30	5	12.40 ± 10.56
			6	18.67 ± 8.39
	0.01	−2.00	4	16.75 ± 11.74
BW 295/C/51	0.00033	−3.48	5	−5.40 ± 12.06
	0.001	−3.00	4	−29.50 ± 35.24
			4	27.50 ± 7.97
			5	31.60 ± 5.42
	0.005	−2.30	6	50.00 ± 6.03
			5	66.60 ± 2.16
	0.01	−2.00	3	56.33 ± 5.46
			4	11.25 ± 7.72
			7	57.43 ± 3.94
	0.05	−1.30	6	73.17 ± 4.05
			6	44.00 ± 8.58
	0.1	−1.00	7	74.00 ± 9.21
	0.5	−0.30	6	67.50 ± 6.44
			5	81.40 ± 2.73
	1.5	0.18	7	79.01 ± 2.7
	3.0	0.48	7	88.43 ± 1.31

main group, it seems possible that seasonal changes in the reactivity of the animals may have had some effect. The substance 295/C/51 is of particular interest, because it affords protection at a lower level (0.001 mg./kg.) than all the other substances investigated, and because the maximum protection lies above 80 per cent, i.e. higher than that given by the stronger group of the other antihistamines. The impression that this drug has, weight for weight, a stronger antihistaminic action than, for instance, promethazine is confirmed by preliminary clinical observations.

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THE EFFECTS OF PROTEOLYTIC ENZYMES ON THE ISOLATED RABBIT INTESTINE*

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Since enzymes have relatively specific substrate requirements, by treating a biologically active substance with a specific enzyme the chemical nature of that substance can often be determined. Thus the disappearance of some kind of activity after enzymic action would be considered as an evidence of the presence of a certain vulnerable structure in a substance. This kind of study is especially useful in determining the chemical nature of substances which have not yet been purified. For instance, the fact that the posterior pituitary hormones lose their activities after tryptic digestion suggests that they are proteins or polypeptides.

In recent years, as crystalline preparations of various proteolytic enzymes have become available and their specific activities have been demonstrated, they have been used in differentiating proteins. Thus Wagtendonk (1948) observed that paramecin lost its capacity to kill *Paramecium aurelia* after being treated with desoxyribonuclease, pepsin, or chymotrypsin, but not after tryptic digestion. He concluded that a nucleoprotein was essential for the activity of paramecin, and that basic aminoacids such as arginine and lysine were probably not necessary constituents of it.

Investigations of this sort have been carried out mainly on non-living substances ; little attempt has been made to apply the same principle to living tissues. The following experiments represent such an effort.

METHOD

A section of rabbit's ileum (the animal having been deprived of food for 24 hours) was suspended in Tyrode solution at 38° C. The volume of the bath was 100 c.c. A frontal writing point was used to record the movement.

Crystalline enzymes obtained from Armour & Co. were used in all experiments. The trypsin and chymotrypsin contained " not more than 50 per cent $MgSO_4$." The weights stated in this paper refer to the preparations as supplied. The weights of acetylcholine chloride and of barium chloride ($BaCl_2.2H_2O$) refer to those of the salts. The proper amounts of various agents, contained in 1 c.c. of Tyrode solution, were pipetted into the bath. They were washed out after about one minute unless otherwise stated.

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RESULTS

The effects of chymotrypsin.—Ten mg. of chymotrypsin (Fig. 1, C) caused an initial depression followed by an increase in the intestinal movement. The former is secondary to the Mg content which alone produced similar depressions (Fig. 4, F), and the latter is probably a result of liberated histamine or related substances (Rocha e Silva, 1943). Twenty minutes later the bath was washed out and more chymotrypsin was added (F). Thirty minutes later the bath was washed out thrice. The spontaneous movement as well as the stimulating actions of acetylcholine and barium, as can be clearly seen, were greatly diminished. In other experiments in which larger doses of chymotrypsin were added, the spontaneous movement disappeared and the stimulating actions of acetylcholine and barium were completely abolished.

Pepsin.—Repeated additions of pepsin (Fig. 2, C and D) had no obvious effect on either the spontaneous movement or the actions of acetylcholine or barium. This was to be expected, as the optimum *p*H for pepsin is about 2, whereas the *p*H of Tyrode solution is about 8.

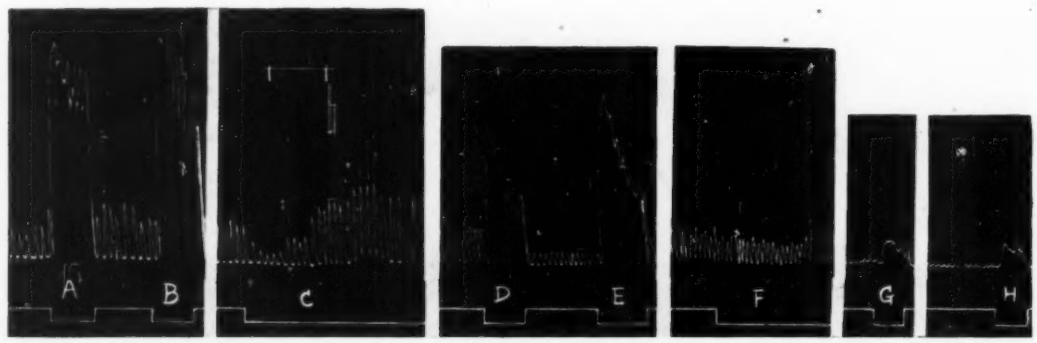

FIG. 1.—Isolated rabbit ileum in 100 c.c. bath. A,D,G—5 μg. acetylcholine. B,E,H—20 mg. barium chloride. C—Chymotrypsin (10 mg.) left in bath for 20 minutes. F—Chymotrypsin (10 mg.) left in bath for 30 minutes.

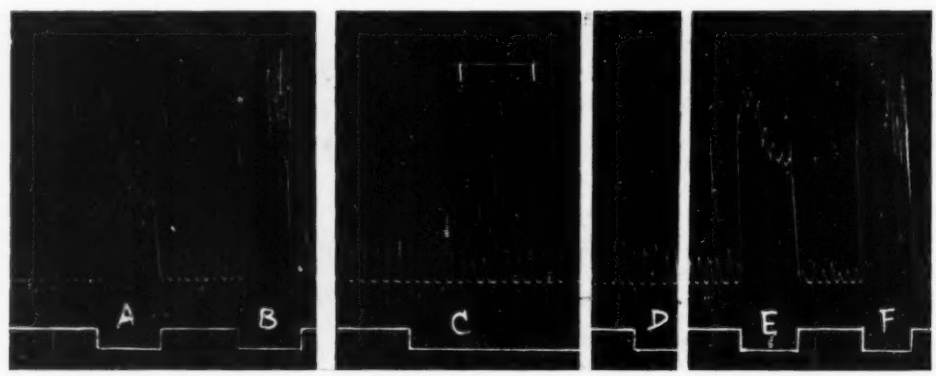

FIG. 2.—Same as Fig. 1. A,E—5 μg. acetylcholine. B,F—25 mg. barium chloride. C,D—Pepsin (10 mg.) left in bath for 30 minutes each.

Trypsin.—The effects of trypsin on the intestinal movement, as shown in Fig. 3 at C (5 mg.) and E (10 mg.), were similar to those of chymotrypsin, an initial depression resulting from its Mg content and a delayed stimulation being probably due to released histamine and related substances (Ramirez de Arellano, Lawton, and Dragstedt, 1946 ; Rocha e Silva, 1940, 1943). Trypsin, however, differed from chymotrypsin in that it diminished greatly the spontaneous movement and the acetylcholine action without affecting the barium action. In some experiments in which 10 to 20 mg. trypsin was added to the bath the spontaneous contraction disappeared and the stimulating action of acetylcholine was completely abolished, while the action of barium was only slightly affected.

Trypsin, inactivated by heating at 60° C. for 1 hour, exerted no effect upon either the spontaneous movement or the intestinal responses to acetylcholine or barium (Fig. 4). The slight depression which occurred immediately after the addition of the enzyme is due to its Mg content, as shown at F in the tracing.

Discussion

The foregoing experiments appear to indicate that chymotrypsin exerts its effects on the intestine by attacking the contractile substance of the smooth muscle. Trypsin, on the other hand, does not seem to affect the contractile substance, since the smooth muscle retains its contractility to barium after trypsin digestion. Thus the abolition of the action of acetylcholine by trypsin tends to indicate the destruction of a receptive substance which is necessary for its action but not for that of

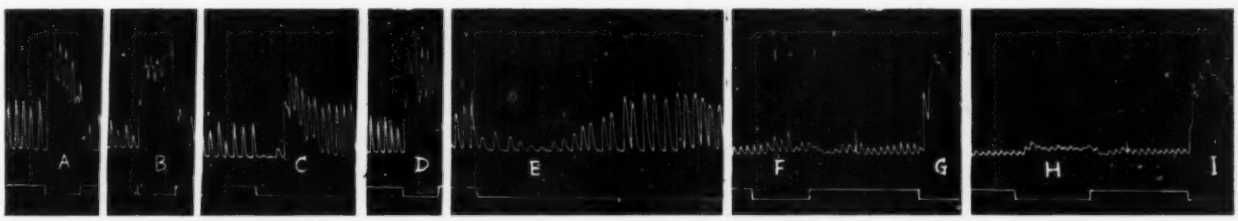

Fig. 3.—Same as Fig. 1. A,G,I—20 mg. barium chloride. B,D,F,H—2 μg. acetylcholine. C—Trypsin (5 mg.) left in bath for 20 minutes. E—Trypsin (10 mg.) left in bath for 30 minutes.

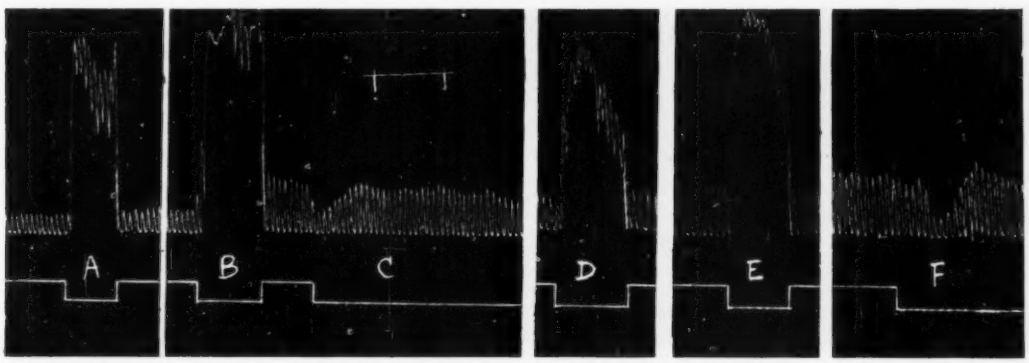

Fig. 4.—Same as Fig. 1. A,D—2 μg. acetylcholine. B,E—20 mg. barium chloride. C—Inactivated trypsin (5 mg.) left in bath for 20 minutes. After washing out, 10 mg. inactivated trypsin was added and left in bath for 30 minutes (not shown in the figure). F—2.5 mg. magnesium sulphate.

barium ; the existence of such a receptive substance has already been suggested by other investigators (Clark, 1937 ; Ing, 1949 ; Langley, 1905 ; Pfeiffer, 1948 ; Welsh, 1948).

If the presence of an acetylcholine receptive substance can be taken as proved, the results described in this paper may be considered as evidence that it is protein in nature. It may be of interest to note in this connection that Welsh (1948) and Welsh and Taub (1951) have suggested acetylcholine receptive substances consist of a family of proteins (enzymes) and that acetylcholine acts as a coenzyme.

Since there appears to be little doubt that the contractile substance is also a protein and is not protected in some way by layers of non-protein structures (as evidenced by its vulnerability to chymotrypsin), its insusceptibility to trypsin is probably a result of the absence at its exposed surface of side groups characteristic for arginine and lysine residues, the presence of which in the substrate has been shown to be essential for the action of trypsin (Bergmann, 1942).

Although proteolytic enzymes similar to trypsin have been found in plasma, the normal functions of the receptive substances are not affected in the intact animal as in the experiments described. This may be because (1) the enzymes are in inactive forms in the plasma under normal conditions (Christensen and MacLeod, 1945 ; Cliffton and Downie, 1950 ; Schwitz, 1937) or (2) the constituents of the body are in a dynamic state, destruction and construction being carried out continuously (Schoenheimer, 1946).

Summary

1. It has been shown that both chymotrypsin and trypsin at suitable doses depress greatly or abolish completely the spontaneous movement of isolated rabbit intestine. Pepsin had no effect under the experimental conditions.

2. It has also been shown that the chymotrypsin-treated intestine loses its responses to both acetylcholine and barium, while trypsin abolishes the action of acetylcholine without significantly affecting that of barium. It is suggested that a receptive substance mediating the action of acetylcholine is destroyed by the tryptic digestion.

The author is greatly indebted to Professors R. L. Stehle and K. I. Melville for their interest during the course of this work.

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REVERSAL OF THE ACTION OF ADRENALINE ON A SINGLE MELANOPHORE IN THE FROG (*RANA TEMPORARIA*)

BY

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Dale (1913) showed that the action of adrenaline on the isolated ferret's uterus was to cause an increase of tone, but if ergotoxine preceded the adrenaline the effect was a decrease of tone. Subsequently, Spaeth and Barbour (1917) found that the melanophores of an isolated fish scale (*Fundulus heteroclitus*), which are normally "contracted" by adrenaline, were "dilated" by adrenaline if they had been previously treated with ergotoxine. Houssay and Ungar (1925), reporting on the action of various drugs (injected into the ventral lymph sac) on the colour of the frog's skin, found that both adrenaline and ergotoxine caused the skin to become lighter in colour, but if adrenaline was given after ergotoxine there was darkening of the skin. Hou (1930) confirmed Spaeth and Barbour's work, and also obtained a similar result using ephedrine in place of adrenaline. Leszczynski (1933) showed that when adrenaline was injected into the amputated legs of a frog the skin became paler, but if ergotamine was injected into the lymph sac before the amputation an adrenaline injection into the amputated legs resulted in the skin becoming darker. Shen (1937) obtained a reversal of the action of adrenaline on the melanophores of the frog's skin with ergotamine, F933, and yohimbine. However, Frommel and Zimmet (1937), using 500 μg. ergotamine and 100 μg. adrenaline injected into the lymph sac of a frog, did not observe any alteration of the action of adrenaline on the skin colour by the previous injection of ergotamine.

Although the melanophores of normal frogs "contract" when exposed to light, pithed or urethanized frogs do so to a much smaller degree (Kobayashi, 1928). Yamaguchi (1929) showed that melanophores are sensitive to temperature changes, and suggested that these responses might obscure the effects of drugs. There are, also, seasonal variations in the response of the frog's blood vessels to adrenaline dosage (Karasek and Poupa, 1937), and this may apply to the sensitivity of melanophores.

It is now generally agreed that the melanophore does not "contract" or "expand," but that the pigment moves; therefore the terms dispersion and concentration are used, rather than dilatation and contraction.

Previous workers did not observe the responses of the same melanophore to both adrenaline and ergotoxine, and therefore the question whether the action of ergo-

toxine was an unmasking of an adrenaline dispersing effect, or an actual reversal of the action of adrenaline, remained unanswered. The present work on the responses of one selected melanophore was undertaken in order to provide an answer to this question.

The effect of ergotoxine on the blood vessels occurs quickly, whereas the response of the melanophore is slow. Doses of ergotoxine large enough to ensure concentration of the melanophore and persistence of action, so that the subsequent effect of adrenaline is reversed, are likely to introduce an error owing to metabolic changes caused by ischaemia (Uyeno, 1922).

Method

The method used was to examine the skin of a frog's web microscopically, using a magnification of 480. A single cell was used for all measurements in a given series, and the measurements made were always along the same axis of the cell. In effect, therefore, the variable recorded is the linear dispersion of the pigment in certain processes of the melanophore. By means of an eyepiece scale these distances were recorded in arbitrary lengths. As occasionally the pigment may flow out and back in a few minutes, measurements were made every minute. Sometimes, when the melanophore is in the concentrated stage the cytoplasmic outline of the cell may be clearly seen, and care was taken to measure the span of the pigment and not of the cell boundary.

The melanophores in the apex of the web just adjoining the digits of the foot give the most reproducible results, and throughout these experiments all observations were carried out on cells in this region.

Pithed frogs were used, the body being supported by a cork platform held by two clamps and the web of one foot fixed to a ring of cork cemented to a glass slide. In order to ensure that the same cell was observed throughout the procedure, the glass slide was clipped to the movable stage of the microscope, and when a suitable area of melanophores was found the vernier scale reading was recorded. Further, the area being observed was marked off and the local distribution of blood vessels recorded by a line diagram. By these methods the frog could be removed and later replaced, the same cell being observed in the same orientation.

The experiments were carried out during the four months of July to October, and the room temperature varied between 17 and 20° C. The background colour was the same, and the intensity of illumination was kept approximately constant. All drugs were injected intravenously by means of a cannula in the anterior abdominal vein.

Ergotoxine (35 μg./10 g. frog) is insufficient to give rise to gross impairment of the peripheral circulation but sufficient for the experimental procedure, provided the subsequent adrenaline injection follows within a minute.

The standard dose for concentration of the pigment was 2 μg. adrenaline (frog weight varied between 18 and 26 g.), and it was always found that 8 minutes later the pigment was concentrated. Thereafter, a slow dispersion occurred.

In each experiment the rate of dispersion was first estimated as the time taken (dispersion time), from the moment of injection of adrenaline, for the melanophore to reach an arbitrary size of 20 scale units (about two-thirds full dispersion).

The following procedures were then carried out to ascertain whether there was an alteration of the dispersion time, measured from the initial 2 μg. adrenaline injection to standard size of 20 scale units.

(1) Adrenaline, 2 μg.; 8.5 minutes later adrenaline, 2 μg.

(2) Adrenaline, 2 μg.; 8 minutes later ergotoxine (35 μg./10 g. of frog); 0.5 minute later adrenaline, 2 μg.

(3) Adrenaline, 2 μg.; 8 minutes later ergotoxine (35 μg./10 g. of frog); 0.5 minute later 0.1 c.c. of saline.

(4) Adrenaline, 2 μg.; 8 minutes later ergometrine (120 μg./10 g. of frog, as this dose was found to be the maximum that could be safely given); 0.5 minute later adrenaline, 2 μg.

The dispersion times in all these procedures are recorded in the Table. Each experiment involving all these procedures was carried out in a random order, and 24 experiments were performed.

Ergometrine has no or little adrenolytic action, and it was used to ascertain whether it would fail, as in mammalian work (except in very high dosage), to reverse the action of adrenaline.

It was found that if the melanophores were submitted to repeated injections of adrenaline, ergotoxine, or ergometrine, the melanophore became concentrated and unresponsive to all drugs. By keeping the frog at 4° C. for a few hours after each procedure, this difficulty was overcome. It was therefore convenient to carry out each procedure in a session, either morning or afternoon, so that a complete experiment lasted 2–3 days. Fortunately, a pithed frog may remain responsive up to 5 days if kept at 4° C.

RESULTS

Fig. 1 illustrates the increased rate of dispersion when the second adrenaline is preceded by ergotoxine.

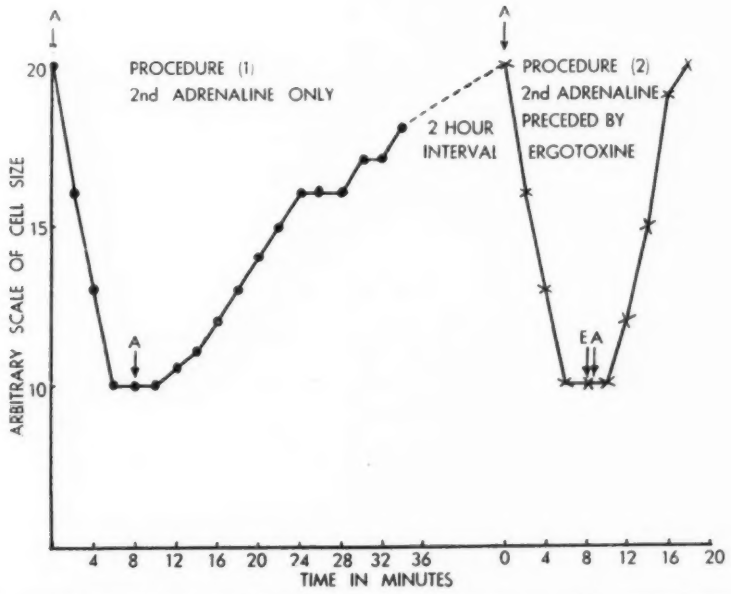

FIG. 1.—The graph illustrates the different rates of dispersion with procedures (1) and (2). A = Adrenaline 2 μg. E = Ergotoxine 35 μg./10 g. of frog.

The results of carrying out all the procedures described, in 24 different frogs, are summarized in the Table.

The mean time for the pigment to reach the arbitrary size of 20 is significantly less when ergotoxine precedes the second adrenaline injection than when ergotoxine

TABLE

TABLE GIVING THE TIME IN MINUTES TO REACH DISPERSION ARBITRARILY FIXED AT 20 BY VARIOUS DRUGS GIVEN 8 MINUTES AFTER INITIAL ADRENALINE

(Number of frogs used: 24)

Drug	Mean time (min.)
Initial adrenaline without further injection	32.1 (S.E.)±1.71
(1) 2nd adrenaline only	32.4 (S.E.)±1.07
(2) Ergotoxine and adrenaline	16.5 (S.E.)±0.55
(3) Ergotoxine only	21.6 (S.E.)±1.14
(4) Ergometrine and adrenaline	40.5 (S.E.)±1.15

Difference between procedures (2) and (3), $P < 0.001$

is given alone. This indicates that the pigment of the melanophore disperses more quickly with adrenaline after ergotoxine than with ergotoxine or adrenaline alone. However, ergotoxine alone also reduces the time taken to reach the arbitrary size of 20. Why ergotoxine alone should cause this dispersion is not clear. If ergotoxine is given when the pigment of the melanophore is dispersed it causes concentration, but if given when the pigment is concentrated, then occasionally a slow dispersion may be seen. Further, under these experimental conditions there may be enough circulating adrenaline after the initial dose to give a modified adrenaline reversal with ergotoxine alone, and this may account for the observed dispersing action of ergotoxine.

It is apparent from the Table that if ergometrine precedes the second adrenaline injection the time taken for the pigment to disperse (size 20) is increased.

In order to expand the scope of the investigation it was decided to repeat the experiment using noradrenaline (a racemic mixture was used). However, it was found that noradrenaline, in a dosage of 2–4 μg. intravenously, caused an initial concentration followed by a relatively rapid dispersion of the pigment.

Discussion

It is suggested that measuring the rate of dispersion of a concentrated melanophore is a suitable method of assessing whether or not a drug has a dispersing action on the melanophore.

The method reported here is open to subjective errors. While this is undoubtedly a serious objection, it is believed that with practice the observational error can be diminished. The objection that keeping frogs for several days may alter the responses of the melanophore is overcome by carrying out the procedures in a random order. The only difficulty encountered in keeping the frogs for several days is that about 25 per cent of them die before completion of the experiment.

Spaeth and Barbour (1917) and Shen (1937), from microscopic observations on the fish scale and the frog's web respectively, concluded that ergotoxine reversed the excitor action of adrenaline. The work reported here on single melanophore responses is in accordance with this previous work. Further, the fact that these observations were made on the same melanophore demonstrates that the mechanism is a reversal of the action of adrenaline and not an unmasking of an adrenaline inhibition.

SUMMARY

A melanophore was initially concentrated by adrenaline and the time taken to reach an arbitrary size was measured linearly by means of a scale in the eyepiece of a microscope. When the melanophore was concentrated, the following separate procedures were carried out: (1) adrenaline alone, (2) adrenaline preceded by ergotoxine, (3) ergotoxine alone, and (4) adrenaline preceded by ergometrine. All the drugs were injected intravenously.

It was found that adrenaline alone did not alter the rate of dispersion, but if ergotoxine preceded the adrenaline then the rate of dispersion was significantly increased when compared to the other procedures.

I should like to thank Mr. K. Sparke for his technical assistance.

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THE RELEASE OF HISTAMINE BY PETHIDINE, ATROPINE, QUININE, AND OTHER DRUGS

BY

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The administration of the potent and specific histamine releasing drug, compound 48/80 (Paton, 1951 ; Feldberg and Paton, 1951), produces a characteristic syndrome of erythema, pruritus, urticaria, and facial swelling in the unanaesthetized dog (Paton and Schachter, 1951). The similarity of this syndrome to the giant oedema and urticaria in so-called drug allergy, or drug idiosyncrasy, suggested the possibility that drugs which cause this acute type of cutaneous reaction in susceptible human subjects might possess primary histamine liberating properties. There is evidence in support of this, since curare is known to be a primary histamine liberator for various animal tissues, as first shown by Alam, Anrep, Barsoum, Talaat, and Wieninger (1939) and by Ambache and Barsoum (1939). These findings have since been confirmed for human skin (Comroe and Dripps, 1946 ; Grob, Lilienthal, and Harvey, 1947). Morphine had been shown to raise the blood histamine concentration in cats by Eichler and Speda (1940), although they failed to attribute this to the release of histamine from tissues. The actual release of histamine from skin and other tissues in this species by morphine and other opium alkaloids, however, has since been demonstrated (Nasmyth and Stewart, 1949 ; Feldberg and Paton, 1951). Atropine has recently been shown to release histamine from the liver of the dog (Burstein and Parrot, 1949). Curare, opium alkaloids, and atropine have been reported to produce cutaneous reactions of the type described above following systemic administration to humans (Bennett, 1940 ; Sollmann, 1948 ; Sutton and Sutton, 1939).

Drugs which are known to produce angio-oedema and urticaria in certain individuals were selected to be tested for histamine releasing properties, particularly in skin. Of these, neoarsphenamine, quinine, and atropine have long been known occasionally to produce dramatic cutaneous reactions after the systemic administration of therapeutic doses (Sutton and Sutton, 1939 ; Sollmann, 1948). More recently, reports have appeared of a similar response to first contact with pethidine (Butler, 1951), and generalized urticaria has been reported after the oral administration of small doses of priscol (Lindquist, 1943). All these drugs were found to release histamine from the isolated perfused skin of the cat, though some were more effective than others. Increased plasma histamine concentrations after injection of the drug into the blood circulation were demonstrable only with pethidine, atropine, and quinine.

Sulphanilamide and dinitrophenol, which are also known to cause similar cutaneous reactions (Sollmann, 1948), failed to release histamine. It is of interest that the clinical evidence indicates that these two drugs appear never to provoke the reaction on first administration (Hageman and Blake, 1937 ; Tainter, Stockton, and Cutting, 1935).

Sodium bromide was tested because of the skin lesions which occur after prolonged bromide therapy. The bromide eruption, however, is considered a toxic effect of bromide accumulation and is not of the " allergic " type (Sollmann, 1948). This drug also failed to release histamine.

The bile salt mixture, sodium tauroglycocholate, was tested because pruritus frequently occurs in obstructive jaundice. This released some histamine from the perfused skin, but much less than the others.

Drugs such as caffeine and alcohol which to our knowledge do not provoke angio-oedema and urticaria were without effect. Hydrochloric acid, isotonic saline, and anoxia likewise failed to release histamine. Distilled water was either ineffective or released barely detectable amounts from the perfused skin.

A preliminary report of some of these results has been published (Schachter, 1951).

METHODS

Cats were anaesthetized with chloralose intravenously, preceded by ethyl chloride-ether induction. Blood samples taken from the femoral artery were heparinized and centrifuged and the plasma tested for histamine. Dogs were anaesthetized with intravenous nembutal. Caffeine was used as base, atropine as sulphate, and quinine as the hydrochloride. Histamine values are given as the base.

Isolated skin preparations.—These were prepared according to the method of Feldberg and Paton (1951), as modified by Feldberg and Schachter (1952). Cat skin preparations weighed 4–8 g. and dog skin preparations 15–20 g. Drugs were injected into the saphenous artery in 0.5 ml. volumes and the perfusate collected from the corresponding vein. The venous outflow ranged between 2 and 3 ml. per min. The infusion was arrested for 10–20 sec. during the injection, permitting the drug to remain in contact with the tissues for this period.

The histamine retained in the subcutaneous oedema fluid of the skin preparation at the end of the experiment was determined by cutting into the oedematous tissue, draining off the fluid, and assaying its histamine content. Since the weight increase after perfusion was known by comparing the weight of perfused skin with that of a similar flap from the normal side, the total histamine in the oedema fluid could be calculated.

Intra-arterial injection in the eviscerated cat.—Drugs were injected into the aorta through a cannula tied into the right renal artery. This assured a high drug concentration in the tissues of the lower part of the animal. The ovarian or testicular vessels were tied and in some cats the adrenal vessels as well. During the arterial injection the inferior vena cava was obstructed for 20 sec. by a loose ligature placed around it above the entrance of the left renal vein. This permitted the injected drug to remain in contact with the tissues for a short time. The left renal vein was tied near the kidney and blood samples for histamine assay obtained with a syringe by inserting a needle through the vein to its junction with the vena cava, which was obstructed by tension on the ligature during this interval. Samples could be obtained repeatedly in this way.

Histamine assay.—All the drugs used, except neoarsphenamine, depressed the response of the guinea-pig ileum to histamine. Their action in this respect roughly paralleled

their histamine releasing ability. Thus the amounts of the various drugs which began to depress the histamine contraction on addition to a 15 ml. bath were, in mg.: pethidine, 0.001 ; atropine, 0.01–0.03 ; quinine, 0.1 ; priscol, 0.1 ; sodium tauroglycocholate, 0.1. The test solution added to the bath in most instances did not contain a sufficient concentration of any of these substances to interfere with the histamine assay. However, when the amount of drug injected was great enough to raise its concentration in the test sample beyond the above values, the standard histamine solutions were made up in the maximum concentration of the specific drug which could be present in the test solution. Histamine assay was performed on the arterial blood pressure of the eviscerated cat when the drug concentration was too great to permit satisfactory assay on the guinea-pig ileum.

Neoarsphenamine increased the response of the guinea-pig ileum to histamine by 20–50 per cent when added to the bath in amounts of 1 mg. or more. It also caused a similar potentiation of the response to acetylcholine.

The fact that the active substance released in each experiment was histamine was substantiated by the abolition of its action on either the isolated guinea-pig ileum or blood pressure of the cat by mepyramine.

RESULTS

Histamine release from isolated perfused skin preparations

Cat skin.—Table I shows the amounts of histamine released from skin preparations by intra-arterial injections of 0.5 ml. volumes of the various drugs. Comparison of histamine releasing potency shows pethidine to be the most effective, followed by atropine, quinine, priscol, neoarsphenamine, and bile salt in this order. Neoarsphenamine occasionally released only small amounts of histamine after a single intra-arterial injection ; on these occasions, a second injection produced the usual release. The possibility that the histamine releasing property of neoarsphenamine was due to oxidized derivatives was excluded by demonstrating that this effect was not less if the drug was carefully dissolved in nitrogenated saline or distilled water and injected immediately, than if the solution was oxygenated and allowed to stand for several hours. Injection of 0.5 ml. volumes of the following substances failed to release any histamine: 10 per cent alcohol (v/v), 5 per cent caffeine, 0.9 per cent sodium bromide, 0.9 per cent sodium chloride, 0.4 per cent sulphanilamide, 0.2 per cent dinitrophenol, and 0.15 per cent hydrochloric acid (w/v). Injection of distilled water occasionally released barely detectable amounts. Anoxia, produced by arresting the inflow of Locke's solution for several minutes, was without effect.

Fig. 1 shows the histamine output from the skin in typical experiments with each drug. The failure of caffeine and ethyl alcohol to release histamine is also shown. The characteristics of the release were identical for pethidine, atropine, quinine, and priscol, in that the venous outflow was temporarily reduced after intra-arterial injection ; by far the greatest histamine concentration was present in the first 10 min. sample and considerable oedema of the preparation was apparent after one hour. The distinctive feature following bile salt injection was the great reduction or arrest of venous outflow, although the inflow from the reservoir continued. Thus, despite the release of lesser amounts of histamine, the development of oedema was rapid and dramatic. The time course of the change in histamine concentration in the perfusate after injection of neoarsphenamine differed from that of the other drugs in so far as the greatest concentrations frequently appeared in later samples, both the initial increase and subsequent decrease in concentration being gradual

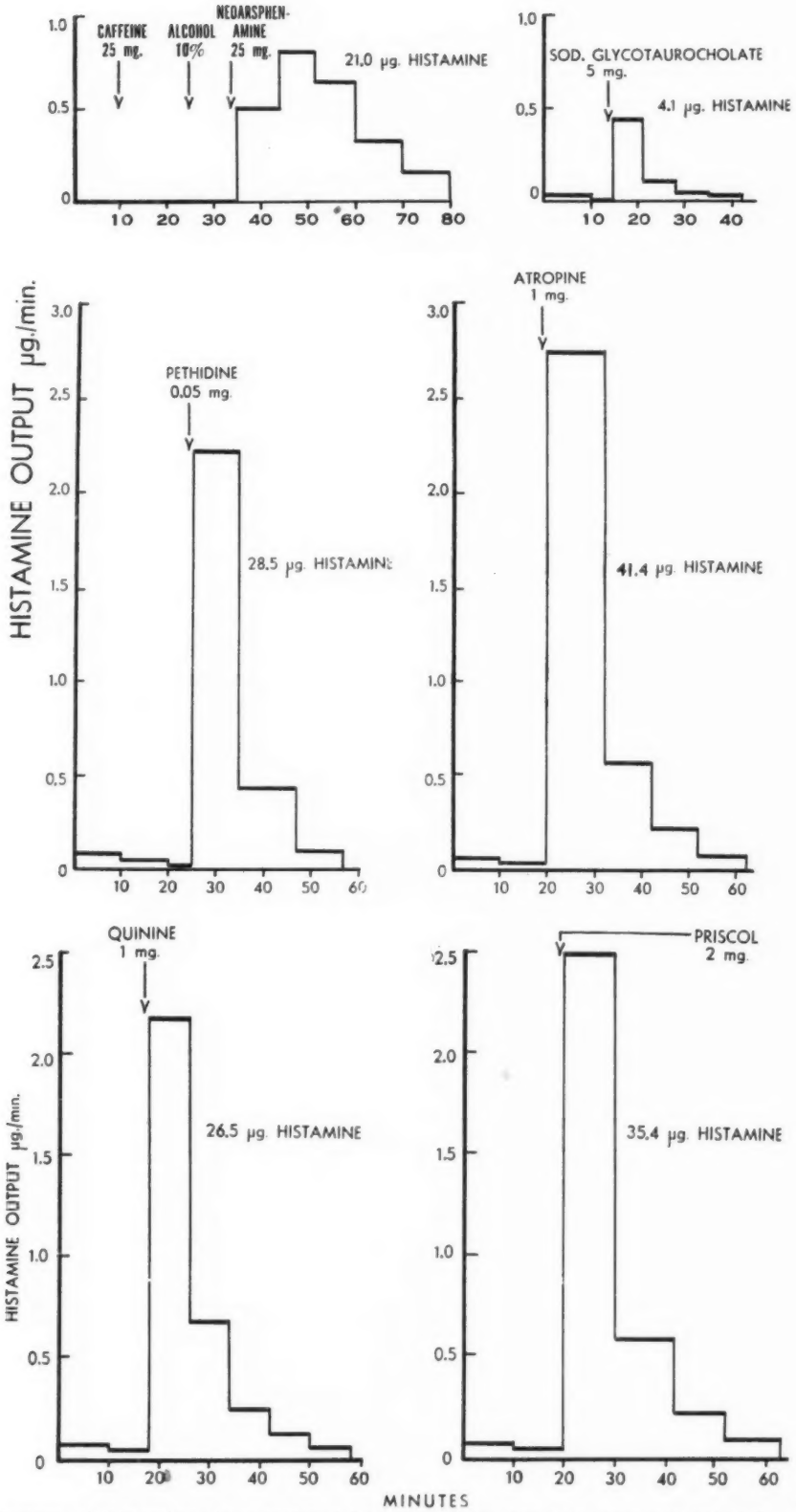

FIG. 1.—Output of histamine from isolated Locke-perfused cat skin preparations following intra-arterial injection of various drugs. Ordinates: histamine output in µg./min. Abscissae: time in 10 min. intervals.

TABLE I

HISTAMINE RELEASE ON INTRA-ARTERIAL INJECTION OF VARIOUS DRUGS IN THE ISOLATED PERFUSED SKIN PREPARATION OF THE CAT

Drug	Amount of drug injected (mg. in. 0.5 ml.)	Total histamine collected in perfusate (μg.)	Total histamine in subcutaneous oedema fluid of isolated preparation (μg.)
Pethidine	25.0	21.0	
	5.0	73.7	6.0
	0.5	55.8	
	0.2	38.3	
	0.05	28.5	2.7
	0.02	0	
	0.01	0	
Atropine	1.0 (2 exps.)	41.4; 30.3	2.8; 3.1
	0.5 (2 exps.)	62.9; 21.8	
	0.1 (2 exps.)	2.9; 2.4	
	0.025	traces	
Quinine	4.0	19.3	4.5
	2.0	36.2	
	1.0	26.5	
	0.1	5.8	
	0.01	traces	
Priscol	2.0	35.4	
	0.5	15.8	
Neoarsphenamine ..	50.0	19.3	19.6
	25.0 (10 exps.)	12.9 (Mean) (S.D. = 6.5)	8.5 (Mean) (S.D. = 6.6)
Sod. glycotaurocholate ..	5.0 (2 exps.)	4.8; 4.1	7.9; 6.5
	4.0	0.6	
	3.0	0.2	3.5

rather than abrupt. Furthermore, the concentrations and total amounts of histamine present in the subcutaneous oedema fluid of the preparation at the end of the experiment were considerably greater after bile salt and neoarsphenamine injections than after the other drugs (Table I).

Dog skin.—Pethidine and atropine, the two most effective drugs for the cat skin, were tested and found effective in releasing histamine from the isolated dog skin preparation. Pethidine was again the more active. The intra-arterial injection of 0.5 mg. pethidine released 21.6 μg. histamine from the skin preparation of one leg. Two mg. atropine released 16.1 μg. from the skin of the opposite leg. The results of these experiments are shown in Fig. 2.

Histamine release by intra-arterial injections in the cat

The substances tested were injected retrogradely into the renal artery of eviscerated cats and blood was collected from the renal vein 2 min. after injection. The drugs thus reached the skin and muscles of the lower half of the animal in higher concentrations than if they were injected intravenously. Pethidine, atropine, and quinine, in this order of effectiveness, raised the plasma histamine concentration

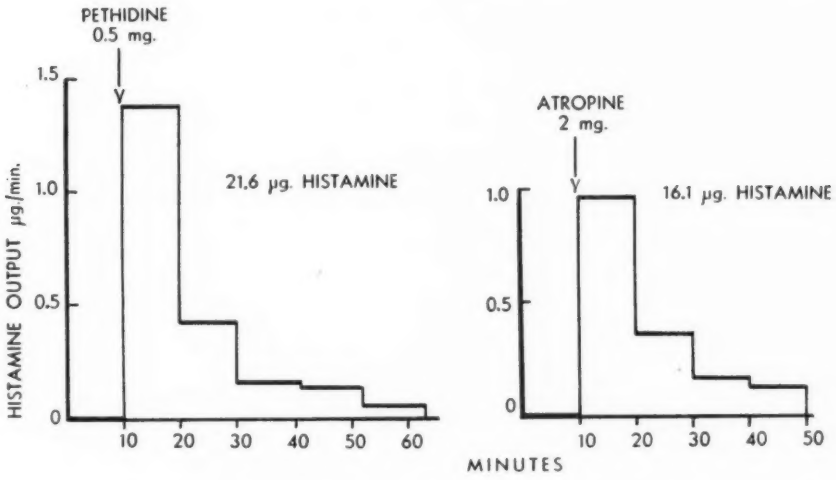

FIG. 2.—Output of histamine by isolated dog skin following intra-arterial injection of pethidine and of atropine.

although greater amounts were required than in the isolated perfused preparation (Table II). Priscol, neoarsphenamine, and bile salt, even with large doses, failed to increase the plasma histamine concentration. This is in contrast to the ready demonstration of histamine release from the isolated Locke-perfused cat skin preparation by these compounds. Plasma histamine assays after pethidine and atropine injections were performed on the blood pressure of the eviscerated cat.

TABLE II

PLASMA HISTAMINE CONCENTRATIONS OF VENOUS BLOOD (INFERIOR VENA CAVA) BEFORE AND 2 MIN. AFTER INJECTION OF PETHIDINE, ATROPINE, OR QUININE INTO THE RENAL ARTERY OF THE EVISCERATED CAT

Drug	Amount of drug injected		Plasma histamine concentration before injection (µg./ml.)	Plasma histamine concentration 2 min. after injection (µg./ml.)
	mg./kg.	mg./ml.		
Pethidine ..	15	15	<0.05	1.4
	15	15	<0.02	0.8
Atropine ..	15	15	<0.03	0.8
	15	15	<0.02	0.15
	15	15	<0.02	<0.02
Quinine ..	50	30	0.01	0.09
	50	30	0.01	0.07
	50	30	0.02	0.02
	25	20	<0.01	0.02
	25	20	<0.01	<0.01
	10	10	<0.02	<0.02

Histamine release by intravenous injections in the cat

Both pethidine and atropine caused an increase in plasma histamine concentration on intravenous injection in the cat. The dosage required, however, produced severe toxic effects, most of which could not be attributed to released histamine.

Pethidine (15 mg./kg.) regularly raised the plasma histamine concentration (Table III), but this dose was sufficient to arrest breathing and artificial ventilation was required. Atropine (20 mg./kg.), after 3 min., raised the plasma histamine concentration from less than 0.01 μg./ml. to 0.05 μg./ml. in one experiment and failed to produce any change in another. These histamine assays were performed on the blood pressure of the eviscerated cat, since the amounts of pethidine or atropine in the plasma were too great to permit assay on the guinea-pig ileum.

TABLE III

PLASMA HISTAMINE CONCENTRATIONS (μG./ML.) BEFORE AND AT VARIOUS INTERVALS AFTER THE INTRAVENOUS INJECTION OF PETHIDINE (15 MG./KG.) IN THE CAT

Exp. No.	Before injection	Minutes after injection			
		3	7	15	40
I	<0.01	0.10	0.10	0.04	0.02
II	<0.01	0.18	0.16	—	0.08
III	<0.04	0.25	0.15	—	0.05

The other drugs failed to raise the plasma histamine concentration under these conditions, even with very large doses. Indeed, a distinct reduction or disappearance of plasma histamine regularly occurred after the intravenous injection of neoarsphenamine (200 mg./kg.) and bile salt (50 mg./kg.). This was associated with a gross increase in size of the buffy cell layer.

DISCUSSION

It has long been known clinically that many drugs, diverse in their pharmacological properties, could evoke a common syndrome bearing considerable resemblance to experimental protein anaphylaxis. This led to the inclusion of this type of drug reaction in the broad category of allergy and its designation as drug allergy. Theoretical objections to this classification were the frequent reactions on first contact, the non-protein nature of the substances, and failure to detect antibodies in reacting subjects. The objection to the non-protein nature of the antigen was subsequently overcome to some extent by the demonstration that the introduction of specific chemical groups into protein molecules did not interfere with the antigenic properties of the protein, and endowed it with a serological specificity related to the nature of the chemical group introduced. This has been shown for many chemical modifications of protein such as iodination (Obermayer and Pick, 1906), nitration (Mutsaars and Gregoire, 1936), and diazotization (Landsteiner, 1945). These experiments led to the concept of haptens or partial antigens, and the hypothesis that if some drugs are repeatedly brought into contact with the tissues a compound antigen is formed which consists of the drug or a part thereof in combination with tissue protein. Such a compound would theoretically derive its specificity from the drug or drug radical and its antigenicity from the combined protein. This hypothesis still fails to explain the facts that there is frequently no evidence of previous contact with the drug incitants and that antibodies are, in general, not demonstrable even by passive transfer (Zinsser and Bayne-Jones, 1939 ; Landsteiner, 1945 ; Sodeman, 1950). The finding, however, that many of these " anaphylactoid "

drugs are primary histamine liberators modifies these theoretical difficulties in so far as prior sensitization need not be regarded as a *sine qua non* of the human reaction. Thus, in addition to reactions in which the subject becomes sensitized to the drug acting as a partial antigen, reactions to first contact could occur on the basis of the primary histamine releasing properties of the drug. It is possible that drugs such as sulphanilamide and dinitrophenol, which do not possess primary histamine releasing properties, evoke reactions only after sensitization. Drugs which possess the primary histamine releasing property might, however, act either on first contact or after sensitization, since experimental animal sensitization to arsenicals (Frei, 1928 ; Sulzberger and Simon, 1934) and quinine (Landsteiner and Chase, 1941) has been demonstrated. The problem why only certain individuals react by releasing histamine from skin on first contact with small doses of these drugs remains. However, the theoretical emphasis could now be placed on the nature of histamine "fixation" in the tissue. At present little is known of the physical or chemical mechanisms stabilizing tissue histamine or permitting its rapid release. Such information might explain the possible "lability" of the skin histamine of individuals who react on first contact with these drugs.

Histamine release was more readily demonstrable from the Locke perfused skin than under conditions of natural blood circulation. The relative effectiveness of the drugs was the same, however, in all three types of preparation employed. The failure to demonstrate increased plasma histamine concentrations in the intact animal with some of the drugs is probably due to a variety of factors such as the histaminase activity of blood, removal or destruction of histamine by various organs, faster circulation rate, or possibly other conditions elevating the threshold for the release mechanism in the presence of blood.

These drugs differ from some other histamine liberators, such as various amines and amidines whose main pharmacological effects are due to the released histamine (MacIntosh and Paton, 1947, 1949 ; Parrot, 1948). In the drugs considered in this analysis, the ability to release histamine is a property incidental to their main pharmacological actions. This is also true of curare and morphine. It is of interest that Burn (1950) has pointed out the overlap in the various pharmacological properties of pethidine, atropine, quinine, and priscol, and suggests that the basis of their common properties is related to their ability to depress the effects of acetylcholine, histamine, and adrenaline. The ability to release histamine can now be added to their common properties.

SUMMARY

1. Pethidine, atropine, quinine, priscol, neoarsphenamine, and bile salt, in this order of effectiveness, release histamine from the isolated perfused cat skin. The characteristics of this release are identical for pethidine, atropine, quinine, and priscol, but differ in some respects for neoarsphenamine and bile salt. Pethidine and atropine were also shown to release histamine from the isolated perfused dog skin.

2. Injection of pethidine, atropine, or quinine into the blood circulation of the anaesthetized cat causes an increase in plasma histamine concentration.

3. The possible significance of these observations to the giant oedema-urticaria type of cutaneous drug reactions in humans is discussed.

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THE ACTION OF *ALPHA-BETA*-DIHYDROXY-*GAMMA*-(2-METHYLPHENOXY)-PROPANE (MYANESIN) ON THE SPINAL CORD OF THE CAT

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Berger and Bradley (1946) first showed that $\alpha\beta$-dihydroxy-γ-(2-methylphenoxy)-propane (Myanesin, Tolserol, Mephenesin) produced muscular relaxation in animals and that it controlled strychnine induced convulsions more effectively than hexobarbitone. They concluded that myanesin acted by " depressing the reflex excitability of the spinal cord." This report led to the use of the drug clinically as a relaxant in anaesthesia (Mallinson, 1947) and in the control of spastic neurological conditions (Schlesinger, Drew, and Wood, 1948). The results were disappointing, because myanesin was either ineffective or transient in its action and because some toxic effects were noted.

Henneman, Kaplan, and Unna (1949), Henneman and Scherrer (1949), and Kaada (1950) studied the effect of the drug on the facilitation and inhibition of the mechanically induced knee jerk caused by electrical stimulation of other parts of the nervous system, such as the brain-stem reticular formation, cerebellum, and cerebrum. Kaada also studied its effect on the complex reflex discharges in the ventral root elicited by electrical stimulation of the dorsal root of the same segment of the spinal cord. Both authors are in general agreement that while myanesin in doses of 25–50 mg./kg. diminishes or abolishes both facilitation and inhibition of the knee jerk resulting from electrical stimulation of other parts of the central nervous system, except in very large doses it has no effect on the knee jerk itself. They concluded that myanesin exerts its effect specifically on the internuncial cells of the central nervous system, apparently at all levels.

This paper reports the results of an analysis of the action of myanesin on the spinal cord of the cat, using the methods developed in previous work on the action of tubocurarine and strychnine on the spinal cord (Bernhard and Taverner, 1951 ; Bernhard, Taverner, and Widén, 1951 ; Taverner, 1952). Monosynaptic and polysynaptic reflex discharges and cord dorsum potentials were evoked by electrical stimulation of appropriate sensory fibres in motor and sensory nerves in unanaesthetized preparations. Special attention has been paid to the blood pressure because myanesin is known to cause a fall in the arterial blood pressure (Berger and Bradley, 1946).

Material and Methods

Cats were used in all experiments. The standard electro-physiological technique employed has been fully described in previous papers. The cord dorsum potentials were recorded by a monopolar silver wire electrode resting on the dorsal surface of the spinal

cord with an indifferent electrode on adjacent muscle. The temperature of the paraffin lake was maintained at 38° C. ± 0.5° C. by means of heaters above and below the preparation. The arterial blood pressure was recorded from the right common carotid artery.

Myanesin, in 1 per cent solution, was injected slowly through an indwelling polythene tube in the left brachial vein. The rate of injection was controlled by direct observation of the blood pressure tracing, but was always 10 mg./kg./min. or less. *d*-Tubocurarine (0.1 per cent) and strychnine hydrochloride (0.01 per cent) were given by the same route. In certain experiments designed to control the influence of blood pressure changes on the preparation, dextran (Berger) was also injected intravenously either to restore the blood pressure to its previous level after the administration of myanesin or to raise the blood pressure above its basal level. Decerebration or spinal section was carried out under ether anaesthesia, but when the experiments were performed two to three hours later its effects had disappeared. Apart from heparin (Evans) 1/10,000 in the blood pressure cannulae, no other drugs were administered to the cats.

Results

Effect on spinal reflexes in unanaesthetized preparations

Stimulation of the low threshold afferent fibres in the gastrocnemius nerve is followed, after an interval of 2.5–3 msec., by a synchronous reflex discharge in the S1 ventral root. The work of Lorente de Nó (1935), Renshaw (1940), and Lloyd (1943) has shown that this discharge represents activity in two neurone (mono-

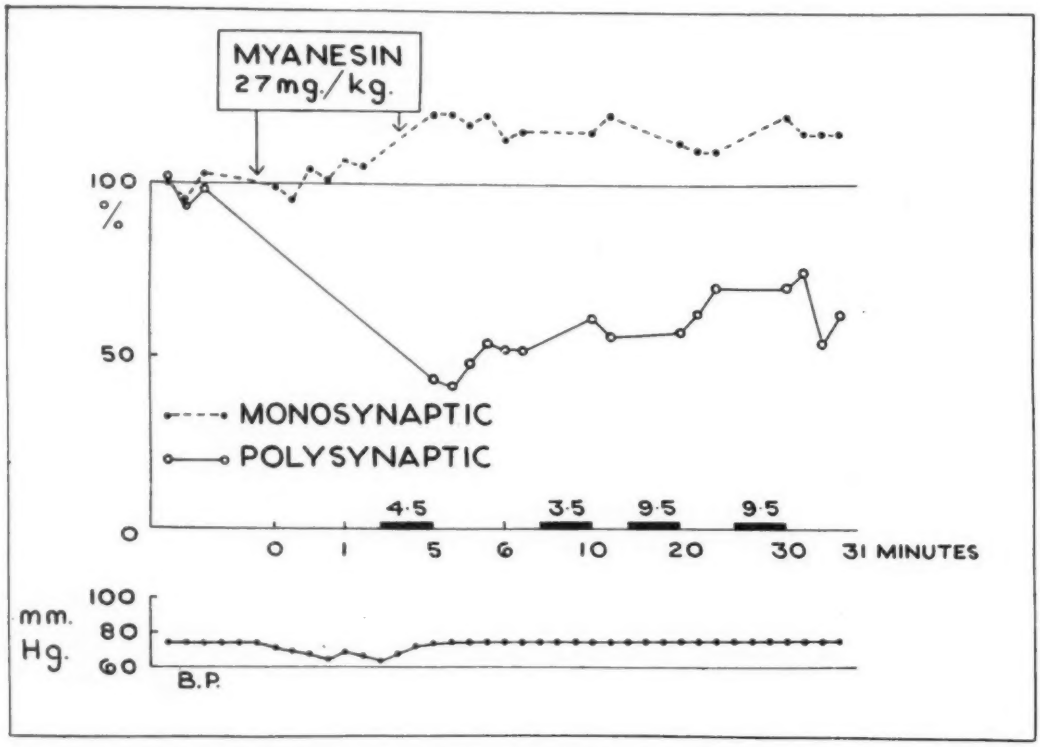

FIG. 1.—Effect of myanesin (27 mg./kg.) on the amplitude of the monosynaptic and polysynaptic reflexes in a decerebrate cat. Ordinates: amplitude as percentage of pre-injection level. Abscissae: time in minutes from start of injection. Bars show periods when recording suspended.

synaptic) arcs and reflects into the muscle from which the afferent fibres arise. This type of reflex arc is the basis of the myotatic reflexes (tendon jerks). If the stimulus is applied to a cutaneous nerve, such as the sural, an asynchronous, irregular discharge appears in the ventral root after 4–5 msec. and persists for 10 msec. or more. Lloyd (1943) has shown that this discharge represents activity in more complex reflex arcs with interpolated internuncial neurones (polysynaptic reflexes) which are the basis of responses such as the flexor and crossed extension reflexes.

Fig. 1 shows the effect of myanesin on the monosynaptic and polysynaptic reflex discharges in a decerebrate cat ; 18 mg./kg. body weight of myanesin had been previously injected and produced a transient and slight fall in the amplitude of the polysynaptic reflex only. Myanesin in a dose of 27 mg./kg. was injected slowly into the left brachial vein and the monosynaptic reflex response was recorded throughout (dotted line). The polysynaptic reflex response was recorded after the injection. Towards the end of the injection the monosynaptic response increased slightly in amplitude and reached a value of 120 per cent of the pre-injection amplitude. This effect on the monosynaptic response is inconstant, but has been seen in all types of preparation, decerebrate, high spinal, and low spinal. Its occurrence seems to depend on the excitability of the preparation and on the dose of myanesin. It usually appears with doses between 20 and 30 mg./kg. In this preparation a further dose of 36 mg./kg. of myanesin was given thirty minutes later, while the monosynaptic response was still augmented. The monosynaptic response fell to its original level during the course of this injection, but returned to its previous level of 120 per cent five minutes later.

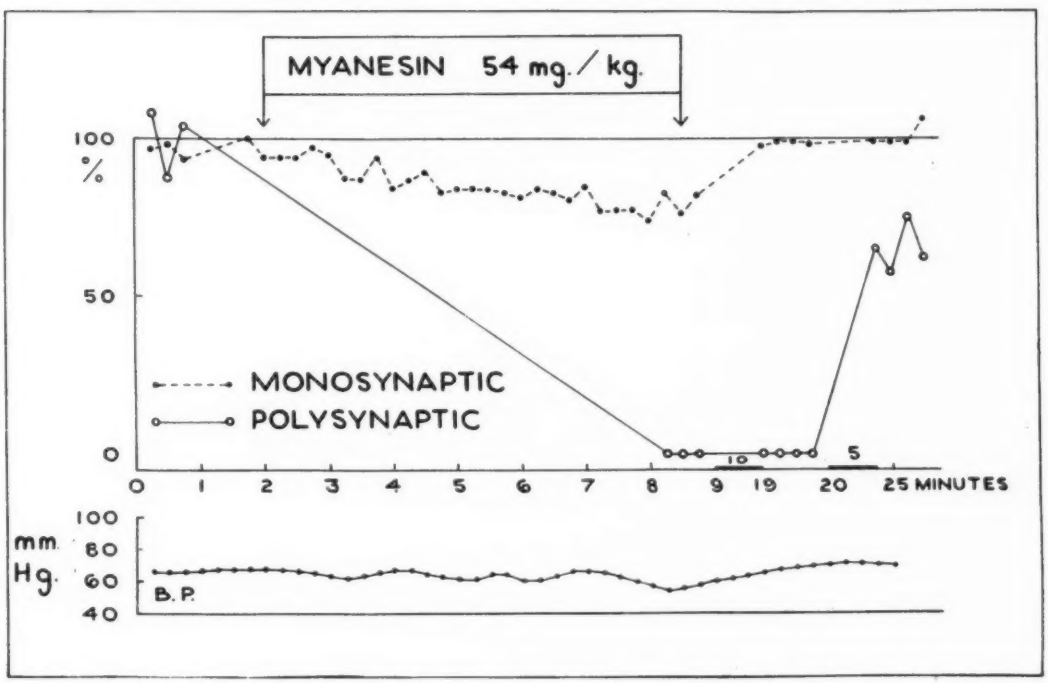

FIG. 2.—Effect of myanesin (54 mg./kg.) on the amplitude of the monosynaptic and polysynaptic reflexes of the same preparation as Fig. 1.

The solid line in Fig. 1 shows the effect of 27 mg./kg. of myanesin on the polysynaptic reflex. Immediately after the injection the response fell to 40 per cent of the pre-injection level. It then began to recover slowly, and thirty minutes later was 75 per cent of the pre-injection level. The next injection of 36 mg./kg. reduced it to 25 per cent of the original level. After this dose the reflex again began to recover but after a longer interval.

Fig. 2 shows the effect of a further injection of 54 mg./kg. of myanesin, making a total of 136 mg./kg. in the same preparation. The monosynaptic response fell to 70 per cent of the pre-injection level during the injection, but fifteen minutes later had attained its previous level. Immediately after the injection the polysynaptic reflex had disappeared ; seventeen minutes later it had reached 75 per cent of the original level.

Fig. 3 shows the effect of the drug on the monosynaptic and polysynaptic reflexes in a decerebrate cat with section of the spinal cord in the upper cervical region ; 21 mg./kg. of myanesin was injected intravenously and the polysynaptic reflex fell to 50 per cent of its original value, but the monosynaptic response was unaffected. The blood pressure remained at 60 mm. Hg during and after the injection, but fell to 50 mm. Hg at a time when the reflexes were normal. It is unlikely that the blood pressure fall is responsible for the reflex changes, because in that event the monosynaptic response would fall in parallel with the polysynaptic response (Taverner, 1952).

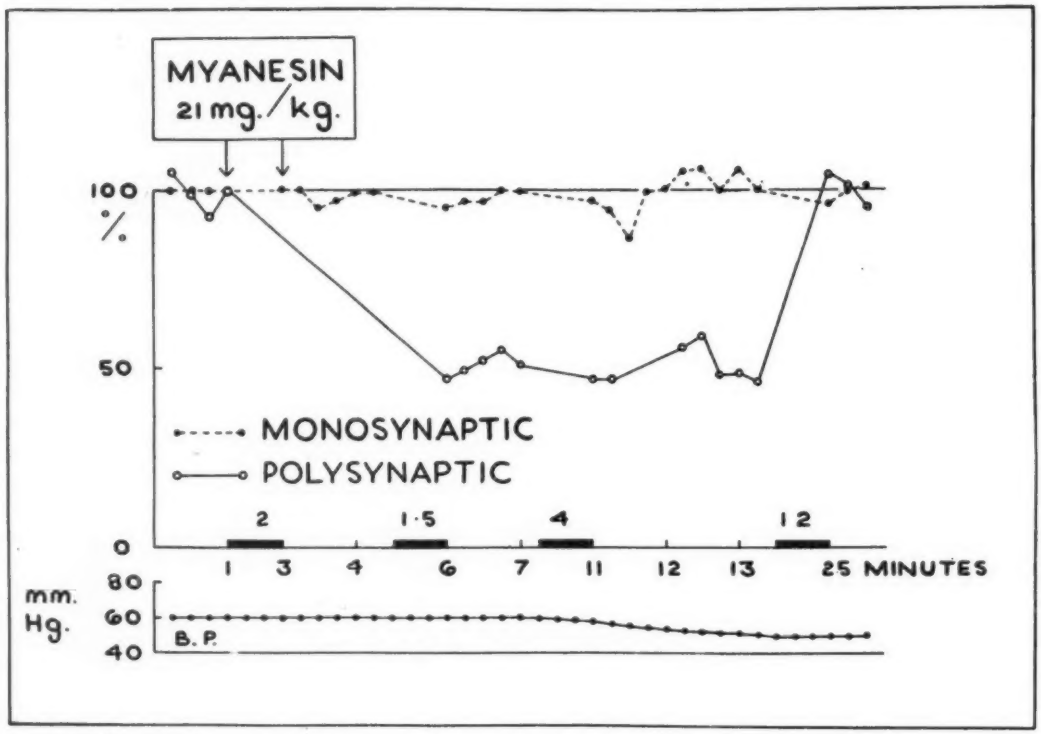

FIG. 3.—Effect of myanesin (21 mg./kg.) on the amplitude of the monosynaptic and polysynaptic reflexes in a decerebrate cat with spinal section in the upper cervical region.

If the spinal cord is severed at the level of T12–L1 segments, the action of myanesin is unchanged. Doses of 15–30 mg./kg. reduce the amplitude of the polysynaptic response to less than 50 per cent, but this action is transient and recovery occurs in 10–20 minutes. The monosynaptic response is unaffected or slightly increased by these doses. Doses of 50 mg./kg. in low spinal preparations depress the polysynaptic response to zero and the monosynaptic response to about 75 per cent of its original value. Recovery begins in both after 10–20 minutes, but is of longer duration than with the smaller doses. Care must be taken with these preparations to avoid depressing the initially low blood pressure of 60 mm. Hg or less to below the critical level of about 40 mm. Hg.

Effect on spinal reflexes in strychninized preparations

It is known that strychnine increases the amplitude of the polysynaptic reflex in all types of preparation. It has recently been shown by Bernhard, Taverner, and Widén (1951) that strychnine increases the amplitude of the monosynaptic reflex in low spinal preparations, but decreases it in high spinal and decerebrate preparations.

The spinal reflexes augmented by strychnine are more sensitive to the action of myanesin than the spinal reflexes in the unanaesthetized non-strychninized preparation. Fig. 4 shows the effect of a small dose of myanesin on the monosynaptic (dotted line) and polysynaptic (continuous line) reflexes in a curarized (1.5 mg. tubocurarine/kg.) low spinal preparation. An injection of strychnine hydrochloride

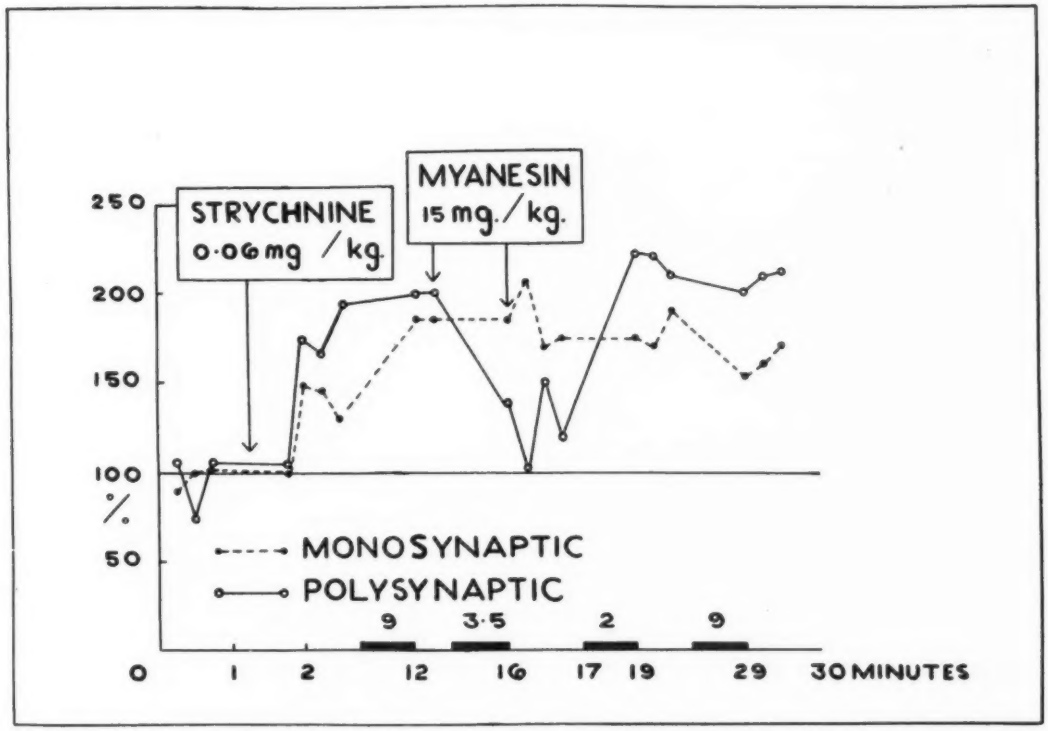

FIG. 4.—Effect of strychnine hydrochloride (0.06 mg./kg.) and myanesin (15 mg./kg.) on the amplitude of the monosynaptic and polysynaptic reflexes in a curarized (1.5 mg./kg.) decerebrate cat with spinal section at the junction of T12–L1 segments.

(0.06 mg./kg.) increased the amplitude of the polysynaptic response to 200 per cent and of the monosynaptic response to 185 per cent of the control level. 15 mg./kg. of myanesin was injected slowly, and temporarily reduced the polysynaptic response to the pre-strychnine control level. Five minutes later the polysynaptic response had returned to its augmented pre-myanesin level. The monosynaptic response was not significantly affected by this injection.

A second injection of 15 mg./kg. of myanesin had the same effect on the two reflexes. Two further injections of 15 mg./kg. reduced both responses to the pre-strychnine level, but ten minutes later they had begun to recover, although they did not attain the levels at first induced by the strychnine. Similar effects were obtained in all types of preparation. Slight depression of the polysynaptic reflex was sometimes observed after only 5 mg./kg of myanesin, but the action of myanesin in strychninized preparations is much more transient than in the non-strychninized cats. Strychnine augmented reflexes can only be kept under control by injections of myanesin repeated at short intervals.

Effect of myanesin on the cord dorsum potentials

It has long been known that electrical stimulation of cutaneous afferent fibres produces potential changes in the spinal cord (Gotch and Horsley, 1891 ; Gasser and Graham, 1933 ; Barron and Matthews, 1938 ; Eccles, 1946 ; Lloyd and McIntyre, 1944 ; Bernhard, 1949 ; and Bremer and Bonnet, 1949). Fig. 6 shows the potential changes recorded by a monopolar electrode on the dorsal surface of the S1 spinal segment after stimulation of the sural nerve. Bernhard (1952) has shown that the N1 potential represents activity in monosynaptically activated cell bodies in the dorsal grey matter, which do not participate in reflex transmission. This negative deflection is followed by an inconstant positivity (P deflection) which originates from propriospinal interneurones extending over several segments of the cord (Bernhard, 1952). This deflection is best seen in preparations with high reflex excitability, but is not essential for reflex transmission.

In a series of experiments on unanaesthetized preparations myanesin was found to have no action upon the cord dorsum potentials except in large doses. Such doses exert a profound influence on the blood pressure, and these changes are probably responsible for any depression of the cord dorsum potentials.

Effect of myanesin on the cord dorsum potentials in strychninized preparations

Bernhard and Koll (1952) found that strychnine produces a marked increase in the amplitude of the P deflection. Larger doses of strychnine produce a succession of regular high voltage waves at frequencies of 10–30 sec. (Bremer, 1941).

The strychnine augmented P deflection is sensitive to myanesin. Fig. 5 shows the effect of the injection of 5 mg./kg. of myanesin into a curarized (1.5 mg./kg.) decerebrate preparation with section of the spinal cord in the upper cervical region. The maximum P deflection after the injection of 0.15 mg./kg. of strychnine hydrochloride is plotted as 100 per cent. Myanesin (5 mg./kg.) caused a prompt fall in the amplitude of the P deflection to 7 per cent of its augmented level. Three minutes later it had increased to 15–25 per cent of its previous level, but there was no further increase. Four further injections of smaller doses of strychnine (0.04–0.1 mg./kg.) rapidly increased the P deflection to its former size. After three of these injections myanesin in a dose of 5 mg./kg. promptly reduced the P deflection to zero and it did

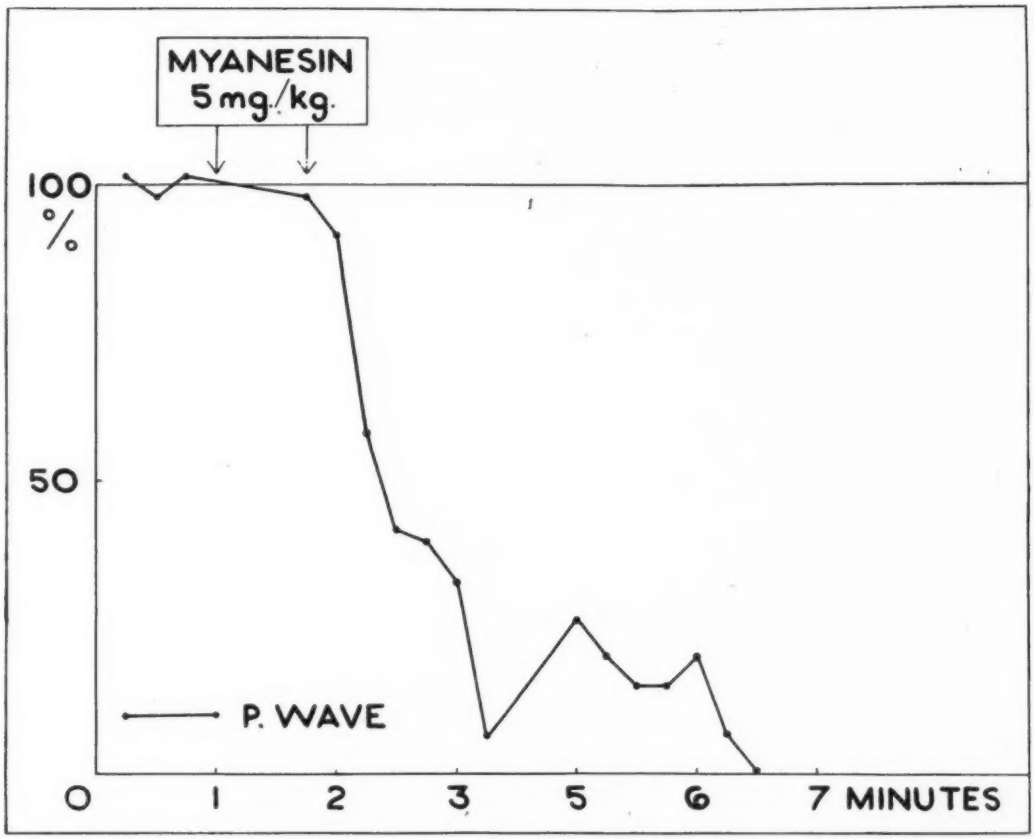

FIG. 5.—Effect of myanesin (5 mg./kg.) on the P component of the cord dorsum potentials elicited by stimulation of the sural nerve in a strychninized (0.15 mg./kg.) and curarized (1.5 mg./kg.) decerebrate cat with spinal section in the upper cervical region. Ordinates: Amplitude of P deflection expressed as maximal P deflection 100 per cent. Abscissae: Time in minutes.

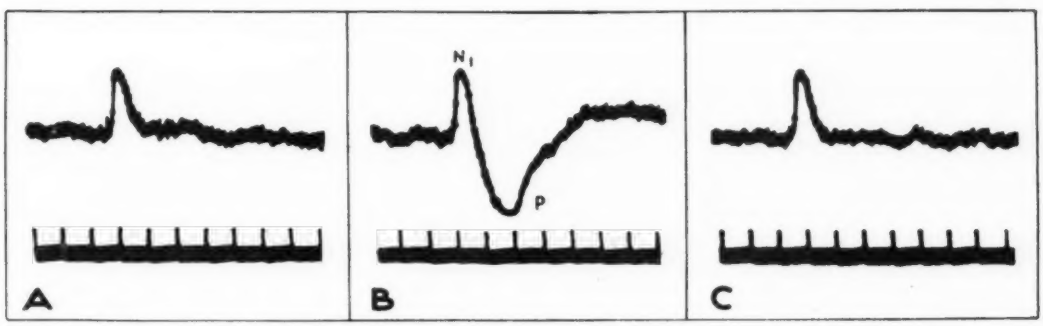

FIG. 6.—Effect of myanesin (5 mg./kg.) on the cord dorsum potentials in the same preparation as Fig. 5. A, Control. B, After strychnine hydrochloride 0.08 mg./kg. C, 3 minutes after myanesin (5 mg./kg.). N: N1 component. P: P deflection. Time: 10 msec.

not reappear until more strychnine was given. Fig. 6 is an extract from the photographic record of this stage of the experiment. Fig. 6A shows the cord dorsum potentials before strychnine, Fig. 6B after 0.08 mg./kg. of strychnine hydrochloride, and Fig. 6C three minutes after 5 mg./kg. of myanesin, when there is no trace of the P deflection. After the fourth injection of strychnine hydrochloride (0.1 mg./kg.) it was found that myanesin in a dose of 2.5 mg./kg. would depress the P deflection, but this action was transient and the P deflection had recovered after two minutes.

It has been shown by Taverner (1952) that strychnine increases the amplitude of the N1 potential to about 125 per cent in low spinal preparations. Myanesin in doses up to 25 mg./kg. in low spinal preparations has no effect on this strychnine augmentation of the N1 potential.

Discussion

The investigation of the action of myanesin on the spinal cord is complicated by the fall of blood pressure which may follow the too rapid intravenous injection of doses of more than 20 mg./kg. body weight. Below a critical level of approximately 40 mm. Hg both the monosynaptic and polysynaptic reflexes diminish in size and may disappear entirely. In the present investigation the drug has been injected slowly and the blood pressure tracing has been inspected throughout. Any fall in the blood pressure has been checked by temporarily stopping the injection. In this way the possibility that the observed changes in the reflexes were due to a fall in blood pressure has been excluded.

The previous investigations by Henneman *et al.* (1949) and Kaada (1950) were mainly directed to the effect of the drug on the facilitation and inhibition of the mechanically induced knee jerk which can be produced by electrical stimulation of other parts of the central nervous system. These authors were in general agreement that myanesin in doses of 25–50 mg./kg. diminished or abolished both facilitation and inhibition of the knee jerk for periods of 30–40 minutes but had no effect on the amplitude of the knee jerk itself. Kaada also reported that myanesin in doses of 10–40 mg./kg. had no effect on the monosynaptic and polysynaptic reflex discharges evoked by electrical stimulation of the mixed afferent fibres in the dorsal root. With doses of 40–60 mg./kg. he produced transient depression of the polysynaptic response, but to reduce the monosynaptic response he had to employ doses of 80–150 mg./kg.

The method of selective study of the monosynaptic and polysynaptic responses employed in this investigation is a more sensitive indicator of the action of myanesin on the spinal cord. The present findings confirm that polysynaptic reflexes are more susceptible than monosynaptic reflexes to the action of myanesin, but in addition they show that the polysynaptic response is temporarily depressed by doses of 20 mg./kg. Doses of approximately 50 mg./kg. abolish the reflex entirely, but it begins to recover within 15 minutes. Throughout the investigation the transient nature of the myanesin effect was striking and this may be of clinical importance.

The present results confirm the findings of Kaada and of Henneman that the monosynaptic response is relatively insensitive to the drug, but transient and slight depression was noted in some preparations with doses of approximately 50 mg./kg. The increase in the amplitude of the monosynaptic reflex which occurs frequently with doses of myanesin of the order of 20–30 mg./kg. is of interest because Henneman

et al. (1949) noted that in one experiment the injection of 10 mg./kg. increased the amplitude of the knee jerk and was followed by the appearance of clonus. They mentioned that similar phenomena have been reported several times in patients. In the present study this effect was observed in both decerebrate and low spinal preparations. It is probably due to the selective blocking of propriospinal interneurones which have an inhibitory effect on the monosynaptic reflex arc.

The anti-strychnine effect of myanesin was described by Berger and Bradley (1946) and confirmed by Kaada (1950), who reported that doses of 25–60 mg./kg. restored the augmented monosynaptic and polysynaptic responses to normal within a few minutes. The present findings provide further confirmation of this, but they show that a dose of only 15 mg./kg. will reduce the strychnine augmented polysynaptic reflex to normal for a few minutes, while doses of 30 mg./kg. have a similar transient effect on the monosynaptic reflex.

An even more striking example of the anti-strychnine effect of myanesin is provided by the studies of its action on the cord dorsum potentials. Doses of 5 mg./kg. of myanesin permanently abolish the strychnine augmentation of the P deflection but leave the originating structure susceptible to the stimulating action of further doses of strychnine. If Bernhard's (1951) view is accepted, that the P deflection represents activity in propriospinal interneurones, the present findings provide evidence for the conclusion of previous workers that myanesin exerts its action on the internuncial cells of the central nervous system. The fact that the drug is equally effective in low spinal preparations with all higher connections severed suggests that its chief site of action is on the propriospinal interneurones. Berger and Bradley (1946) have shown that myanesin has no action on the neuromuscular junctions in the doses employed in the present experiments.

Clinically, myanesin has been disappointing in the treatment of spastic states, extrapyramidal disorders, and tetanus (Schlesinger *et al.*, 1948 ; Bickers *et al.*, 1950 ; Ablett, 1952). It is evident that the doses used clinically could at best have only a transient effect on such conditions and under certain circumstances might even make them worse. The possibility of using continuous intravenous infusions of low concentrations of myanesin in the treatment of convulsive states produced by strychnine or tetanus toxin appears worthy of further consideration, but the hypotensive action of the drug may render this method impracticable.

Summary

1. The action of $\alpha\beta$-dihydroxy-γ-(2-methylphenoxy)-propane (myanesin) on the monosynaptic and polysynaptic reflexes and the cord dorsum potentials elicited by selective electrical stimulation of appropriate afferent fibres, in unanaesthetized cats before and after strychnine, has been investigated. Special attention has been paid to changes in arterial blood pressure produced by the drug.

2. The results of previous workers have been in general confirmed, but it has been shown that myanesin produces transient depressions of the polysynaptic reflex in smaller doses than previously reported.

3. Under certain conditions small doses of myanesin increase the amplitude of the monosynaptic reflex.

4. The anti-strychnine effect of myanesin has been confirmed, but it has been shown that the drug is effective against both the monosynaptic and polysynaptic reflexes in smaller doses than previously reported.

5. The action of myanesin on the cord dorsum potentials is described. In very small doses the drug permanently abolishes the strychnine augmentation of the P deflection.

6. The significance of the results is discussed.

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THE ACTION OF NICOTINE ON THE CILIARY GANGLION

BY

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(Received June 7, 1952)

The existing conception of the peripheral autonomic nervous system is that due to Gaskell (1916), who supposed that the ganglia of the cranio-sacral division lie within or close to the organ they innervate and have short fibres which represent the postganglionic effector element. The sympathetic ganglia, on the other hand, lie outside the organ they supply, and have long easily distinguishable postganglionic fibres. In general, Dale's classification of the two systems according to the humoral transmitter liberated at postganglionic nerve endings agrees with Gaskell's anatomical distinction, but certain exceptions of cholinergic fibres running within the sympathetic system are known. Evidence that there are adrenergic fibres running in the parasympathetic system has been put forward by Ambache (1951) and Ambache and Edwards (1951), who noted that in the isolated intestine, after the parasympathetic elements have been exposed to the action of botulinum toxin or atropine, nicotine produces an inhibition of movement instead of the contraction elicited before. This action of nicotine is abolished by hexamethonium or ephedrine, and the authors suggested that there are present in the intestine itself, within the parasympathetic plexus, ganglia having short adrenergic postganglionic fibres which inhibit intestinal movements. Kottegoda (unpublished observations), working in this laboratory, finds that nicotine exerts on isolated rabbit auricles an inhibitory action followed by a stimulant action. After atropine has been given to paralyse the cholinergic elements, nicotine has only a stimulant action, and this is abolished by hexamethonium. He suggests that ganglia having adrenergic postganglionic fibres are also present in heart muscle. These observations indicate, therefore, that preganglionic fibres within the parasympathetic system terminate in or near the organ not only at ganglion cells having cholinergic postganglionic fibres, but at others having adrenergic fibres.

Dale and Laidlaw (1912) found that nicotine produced a dilatation of the cat's pupil after the corresponding superior cervical ganglion had been removed and the suprarenal glands excluded from the circulation. They considered that this effect was not due to a direct action of nicotine on the iris muscle, because the phenomenon could not be demonstrated *in vitro*. Nor did they think the dilator effect could be produced by the liberation of an adrenaline-like substance from deposits of chromaffin tissue in the sympathetic system, since exclusion from the circulation of all but a small portion of the sympathetic system by ligating the aorta at the diaphragm did not reduce the effect.

There remains the possibility, therefore, that nicotine acts on the iris muscle indirectly through the ciliary ganglion. By analogy with the experiments on the isolated intestine and auricles cited above, it seems reasonable to suppose that the

ciliary ganglion may contain cells having adrenergic postganglionic fibres which are stimulated by nicotine to produce a dilatation of the pupil. The experiments described below were designed to test this hypothesis.

METHOD

In cats under chloralose anaesthesia the right ciliary ganglion was removed according to the technique of Shen and Cannon (1936). An incision about 3 cm. long was made extending from the auditory meatus towards the eye, and sufficient of the orbit was chipped away to open the sheath and make dissection behind the eye possible. The external rectus muscle was retracted dorsally, and the ciliary ganglion found by tracing centrally the branch of the third nerve supplying the inferior oblique muscle. After removal of the ciliary ganglion both superior cervical ganglia were removed, and the suprarenal glands were tied off. The right pupil will be referred to as the denervated pupil, and the left pupil with ciliary ganglion intact as the innervated pupil. The nictitating membranes were fastened back and the eyelids slit to expose the pupils as completely as possible. Pupil size was recorded photographically, a control picture being taken before each injection, and thereafter at 1 min. intervals starting 30 sec. after the injection. Drugs were injected into the femoral vein.

RESULTS

The resting diameter of the innervated pupil was a slit, and that of the denervated pupil 10–16 mm. (Fig. 1). In each of 17 cats 2 mg. nicotine base produced a

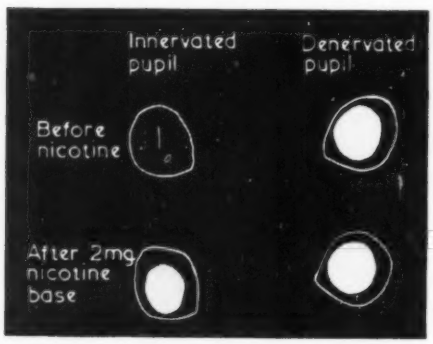

FIG. 1.—Drawings made from photographs showing the effect on the cat's pupil of intravenous nicotine. The innervated pupil has only the parasympathetic nerve supply intact.

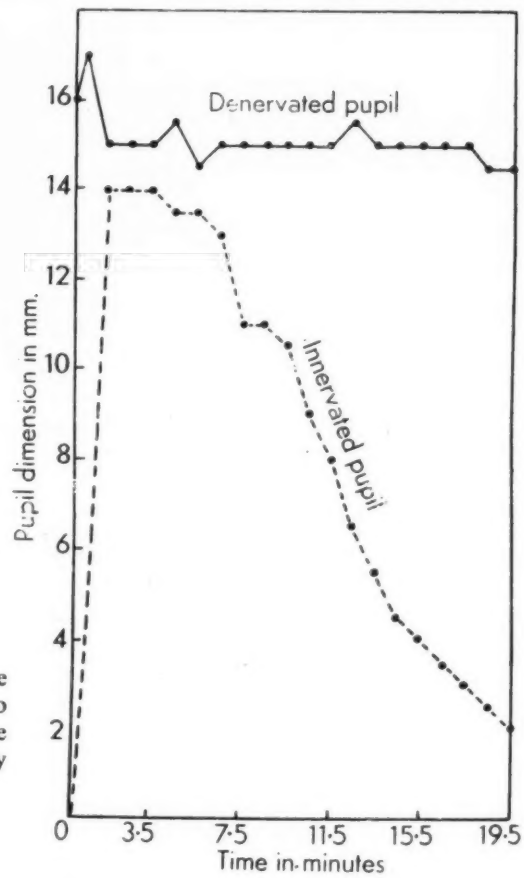

FIG. 2.—Graph showing the effect of nicotine (2 mg. base injected intravenously at zero time) on the denervated pupil and on the pupil having a parasympathetic nerve supply only, in a cat.

dilatation of the innervated pupil which disappeared slowly in about 20 min. (Figs. 1 and 2). In 5 cats there was a dilatation of 1–2 mm. in the denervated pupil which passed off in 2 min., but in the remaining 12 cats there was no effect on this pupil. Spontaneous fluctuations of about 0.5 mm. were often seen in the size of both pupils.

In 3 cats the innervated pupil was kept in shadow and was not therefore maximally constricted. Nicotine, in doses ranging from 0.1–2 mg. base, produced no trace of constriction, and above the threshold dose the only effect observed was dilatation. In one of these cats the threshold dose of nicotine was 0.5 mg., which produced a very small dilatation lasting 30 sec.; 0.6 mg. had the same effect; 0.7 mg., however, produced a dilatation of 4 mm. lasting for 16 min.

In contrast to nicotine, intravenous adrenaline produced consistently a transitory dilatation of both pupils, thus showing that the iris muscle would be sensitive to adrenaline liberated at the nerve endings by stimulation of sympathetic elements.

In 8 experiments, 1.25 mg. hexamethonium bromide was given to paralyse the ciliary ganglion, and this produced a dilatation of the innervated pupil which lasted 30 min. Nicotine given after hexamethonium had no effect, except in 2 cats in which there was a brief constriction of the innervated pupil. Adrenaline, however, still produced a dilatation.

In order to eliminate the influence of the cholinergic nerves on the pupil, 4 mg. atropine was injected intravenously, and this caused the innervated pupil to dilate. Again there was room for further dilatation, but nicotine now had no dilator action on the pupil.

Ergotoxine ethanesulphonate (B.P.1932) was administered in doses sufficient to reverse the action of adrenaline on the blood pressure, thus eliminating possible sympathetic effects in the pupil. This resulted in a steady diminution in the size of the denervated pupil. In the innervated pupil nicotine still produced a dilatation, and, although this effect was smaller than before, it lasted for the same length of time (20 min.) (Fig. 3). Adrenaline had no effect on either pupil.

In one experiment the phenyl ether of choline (Hey, 1952) produced in both pupils a dilatation which was abolished by hexamethonium bromide, and dilated

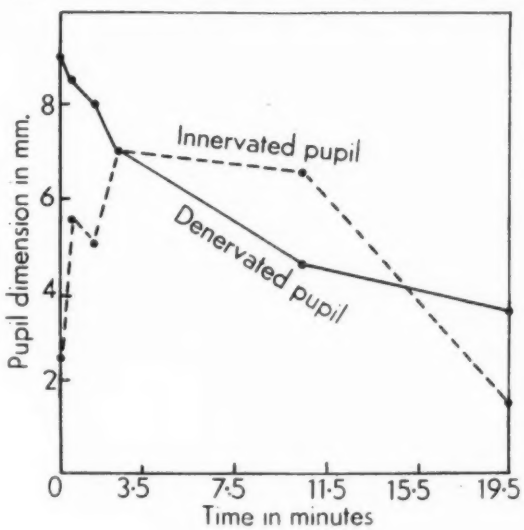

FIG. 3.—Graph showing the effect of nicotine (2 mg. base injected intravenously at zero time) on the cat's pupil after ergotoxine (5 mg.). The innervated pupil has an intact parasympathetic supply only.

the isolated pupil. This substance also produced a large rise in the cat's blood pressure when several large doses of nicotine had been given previously and 15 mg. of nicotine had no effect. Tubocurarine (3–5 mg.) in one experiment dilated both pupils, and the effect on the innervated pupil lasted for the rest of the experiment.

In four additional experiments nicotine (1 g. base/100 ml.) painted on the ciliary ganglion produced a dilatation of the pupil, and this effect was observed whether the superior cervical ganglia and suprarenal glands were intact or excised. In the eserinized animal acetylcholine (1 g. chloride/100 ml.) produced similar effects. Stimulation of the 3rd nerve caused the pupil to constrict, and this effect was blocked by painting the ciliary ganglion with nicotine or in the eserinized animal with acetylcholine. After section of the 3rd nerve, neither nicotine nor acetylcholine painted on the ganglion had any effect on the pupil.

In experiments on the isolated iris in which temperature and *p*H were carefully controlled, nicotine had no effect on pupil size except in very high concentrations (3 mg./ml.) when the effect could not be considered as specific.

Discussion

These experiments indicate that the dilator action of nicotine on the pupil after sympathetic denervation is manifest for the most part through the ciliary ganglion, and the small effects sometimes seen on the completely denervated pupil might be due to a few stray ganglion cells remaining after removal of the ciliary ganglion. It seems clear that the main dilator action of nicotine is no longer seen after removal of the ciliary ganglion, or after paralysing the ciliary ganglion with hexamethonium bromide.

If we assume that, after removal of the superior cervical ganglion, nicotine acts solely through the ciliary ganglion, there are two ways in which it might produce its effects: (1) by stimulation of cells having adrenergic postganglionic fibres; (2) by paralysis of the cholinergic elements, which would result in a passive dilatation of the pupil. Adrenaline produces only a very transient dilatation; thus, if nicotine acts by stimulating adrenergic elements, its effect would last for 20 min. until the pupil dilatation had worn off. This seems unlikely, since the stimulant action of nicotine on sympathetic ganglia, as recorded on the blood pressure, lasts for only a few minutes. Hexamethonium bromide, which, so far as is known, has no ganglion-stimulating properties, produces similar effects to nicotine, and the evidence suggests that these two drugs produce their effects on the pupil by a paralytic action on the cholinergic elements in the ciliary ganglion.

After atropine in doses sufficient to block the cholinergic mechanism, nicotine produces no dilatation, which indicates that there are no active adrenergic elements present, since these would be unaffected by atropine. After adrenaline-like substances had been antagonized with ergotoxine, nicotine still had a dilator effect on the innervated pupil. The dilatation was less after ergotoxine, probably because of its direct constrictor action on the iris muscle seen in the right pupil, which would affect the degree of dilatation after nicotine, but not the duration.

Phenyl ether of choline does not yield any information with regard to the action of nicotine-like drugs on the ciliary ganglion, since it has sympathomimetic properties.

The fact that the innervated pupil, which is not fully constricted in the first place, does not show any trace of constriction after small doses of nicotine suggests that

nicotine has no stimulant action on the cells of the ciliary ganglion. This hypothesis would explain why section of the third nerve abolishes the effects of nicotine painted on the ciliary ganglion. If the only action of nicotine on the ganglion is a paralytic effect which blocks impulses coming from the brain, then when these impulses have already been cut off nicotine can have no further effect. There is no evidence that nicotine stimulates the cells of the ciliary ganglion under these conditions.

The experiments reported above thus produce no evidence to indicate the presence in the ciliary ganglion of cells having adrenergic postganglionic fibres, and one might conclude, therefore, that all the cells contained in the ganglion have cholinergic postganglionic fibres.

Summary

1. In cats the effect of nicotine on the pupils was recorded after removal of the right ciliary ganglion, both superior cervical ganglia, and the suprarenal glands.

2. Nicotine produced in the pupil with ciliary ganglion intact a dilatation lasting 20 min. in all 17 cats used, and in the denervated pupil a small transient dilatation in 5 of the cats. Both effects were abolished by hexamethonium bromide.

3. After ergotoxine, nicotine still produced a dilatation of the innervated pupil, but after atropine nicotine had no effect.

4. Hexamethonium bromide had similar effects to nicotine and dilated only the pupil with ciliary ganglion intact.

5. Nicotine and, in the eserinized animal, acetylcholine painted on the ciliary ganglion dilated the pupil and blocked the constrictor response to stimulation of the 3rd nerve. After section of the 3rd nerve, nicotine and acetylcholine painted on the ganglion had no effect.

6. It is concluded that the dilator effect of nicotine on the cat's pupil deprived of the sympathetic nerve supply is produced entirely by a paralytic action on the cholinergic nerve elements in the ciliary ganglion which results in a passive dilatation of the pupil. There is no evidence to indicate that some cells in the ciliary ganglion have adrenergic postganglionic fibres.

I should like to thank Professor J. H. Burn and Dr. E. Bülbring for their help and advice with this work.

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Note added in proof.—Since this paper was submitted, W. L. M. Perry and J. Talesnik have given a communication at the July, 1952, meeting of the British Pharmacological Society in Edinburgh in which they reported that nicotine, injected into the lingual artery of the cat, produces a constriction of the pupil; this occurred after section of the third nerve and was apparently due to a stimulant action of nicotine on the cells of the ciliary ganglion. It remains, however, to be explained why nicotine, which stimulates a sympathetic ganglion such as the superior cervical ganglion, both on intravenous injection and on direct application, fails to stimulate the ciliary ganglion when applied in these ways.

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CHOLINESTERASE IN THE IRIS AFTER REMOVAL OF THE CILIARY GANGLION

BY

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When tissues are denervated, they often become more sensitive to chemical stimuli, though to some substances they become less sensitive; thus the cat's iris becomes more sensitive to adrenaline, but does not respond to tyramine at all (Burn and Tainter, 1931). The increase in sensitivity has not yet been explained; it is probably due to several factors. One factor may be a fall in the amount of enzyme near the nerve ending, but until recently the only evidence of such a fall in the autonomic system was the fall in cholinesterase observed by v. Brücke (1937) in the superior cervical ganglion after section of the preganglionic fibres. It has now been shown (Burn and Robinson, 1952) that when the superior cervical ganglion is removed in the cat there is a fall in the amount of amine oxidase in the nictitating membrane, and likewise that when the stellate ganglion is removed there is a fall in the amount of amine oxidase in the arteries of the cat's foreleg. In view of the evidence of Shen and Cannon (1936) that the iris of the cat's eye becomes more sensitive to acetylcholine after removal of the ciliary ganglion, experiments have been carried out to see if there is a fall in the amount of cholinesterase in the iris after this operation.

METHOD

The right ciliary ganglion was removed in a series of 14 cats under pentobarbitone anaesthesia. In extirpating the ganglion an incision was made through the skin from the auditory meatus almost to the outer junction of the eyelids. Portions of the zygoma were removed, and access to the post-orbital space was then readily obtained. The external eye muscles were retracted, and after some practice the ganglion was found without difficulty. The cats were killed at 2, 6–8, 21, or 35 days later with coal gas. Both irises were removed immediately and stored at –15° C. until estimations were carried out. Quantitative determinations of cholinesterase were made manometrically by Warburg's technique, using 0.2 M-acetylcholine, 0.3 M-acetyl-β-methylcholine, and 0.06 M-benzoylcholine as substrates. Each iris was weighed and a suspension was made by cutting the tissue with scissors, freezing in a mortar at –15° C., and then pounding; 3 ml. of bicarbonate Ringer was added to each iris. For the manometric experiments conical flasks with one side bulb were used, and in each experiment a "substrate blank" was set up. When benzoylcholine was the substrate 1.3 ml. extract and 1.1 ml. bicarbonate Ringer were put into the flask. With the other substrates 0.5 ml. extract and 1.9 ml. bicarbonate Ringer were used, as the enzyme activity was greater; 0.6 ml. substrate was put into the side tube. The flasks were filled with nitrogen and carbon dioxide and incubated at 37.5° C. Under these conditions the carbon dioxide evolution of the substrate without enzyme was small. Readings were taken every 5 min. for 30 min., starting 3 min. after

tipping. The carbon dioxide evolution was plotted on a graph against the time, and the enzyme activity calculated from the reading at 20 min. and expressed as ml. CO_2 evolved/g. of tissue/hr.

Results

The general result of the observations was that the removal of the ganglion was found to cause a fall in the cholinesterase in the iris. Details of the estimations with the three substrates are given in Table I. In all observations except two the figures for cholinesterase in the denervated iris were less than those for the corresponding

TABLE I

ESTIMATION OF CHOLINESTERASE IN CAT IRIS

Time in days after denervation	Cholinesterase activities expressed as ml. CO_2/g./hr. for different substrates					
	Acetylcholine		Acetyl-β-methylcholine		Benzoylcholine	
	Normal	Denervated	Normal	Denervated	Normal	Denervated
2	4.85	4.08	4.15	3.69	0.20	0.19
	12.8	10.0	11.9	8.5	0.66	0.49
	12.2	13.4	9.72	8.63	0.61	0.58
6–8	—	—	5.66	3.17	0.46	0.32
	5.71	3.28	4.04	2.49	0.21	0.24
	9.03	5.52	8.27	4.65	0.58	0.52
21	—	—	5.2	2.17	0.33	0.29
	5.79	2.75	5.01	1.98	0.39	0.33
	14.9	6.93	14.9	5.87	0.76	0.55
35	—	—	2.00	1.26	0.47	0.37
	—	—	5.28	2.27	0.44	0.37
	9.67	3.21	6.51	2.00	0.50	0.31
	7.90	2.74	6.62	1.28	0.37	0.31
	8.14	3.46	7.15	2.33	0.66	0.46

normal iris. The exceptions were the third figures when acetylcholine was used as substrate on the second day after denervation, and the second figures when benzoylcholine was used as substrate 6–8 days after denervation.

The result in each denervated iris was calculated as a percentage of the result in the corresponding normal iris, and a mean figure for each substrate at a given time after denervation was then obtained. The mean percentages have been plotted in Fig. 1, from which it appears that the percentage fall when acetylcholine was used as a substrate was almost identical with that when acetyl-β-methylcholine was used. The percentage fall when benzoylcholine was used was much less.

Discussion

The results obtained furnish another illustration, in addition to those already mentioned, that when an autonomic nerve fibre degenerates there is a decrease in the amount of the enzyme which is normally engaged in destroying the transmitter. It seems very probable that when this decrease in the enzyme is demonstrable the increase in sensitivity of the tissue to the transmitter is, at least in part, due to the decrease. An increase in the sensitivity of the cat's iris to acetylcholine has been

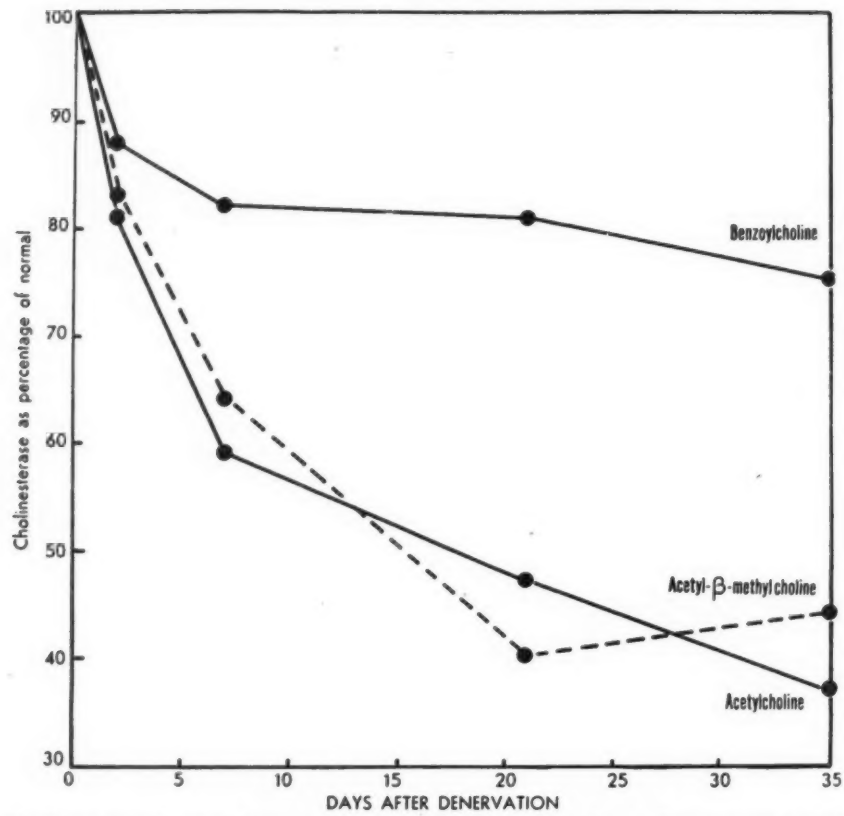

FIG. 1.—Cholinesterase in the denervated iris of the cat some days after removal of the ciliary ganglion. The ordinate is the amount in the denervated iris expressed as a percentage of that in the corresponding normal iris. Each point is the mean value for 2–5 cats.

described by Shen and Cannon (1936) and also by Keil and Root (1941) to follow the extirpation of the ciliary ganglion. (The observations of Shen and Cannon have been described by Cannon and Rosenblueth, 1949.) Shen and Cannon made their observations in cats in which one ciliary ganglion was removed two weeks previously and the remaining ciliary ganglion and both superior cervical ganglia were removed acutely. Eserine was instilled into the conjunctival sac of both eyes followed by acetylcholine applied in the same way. The pupil of the acutely denervated eye gradually constricted in the course of one hour, while the pupil of the eye from which the ciliary ganglion was removed two weeks previously passed through a phase of extreme constriction, maximal in thirty minutes, and diminishing to equal the constriction in the other eye at the end of one hour. Presumably the constriction in the acutely denervated eye was due to eserine prolonging the action of the transmitter, and that in the eye denervated two weeks before was due to hypersensitivity to the acetylcholine instilled. Keil and Root described increased sensitivity after section of the preganglionic fibres to the ciliary ganglion or extirpation of the ganglion itself. They determined the reaction of the iris by intravenous injection into the unanaesthetized cat of a dose of 5 mg./kg. of acetylcholine, and observed that the sensitivity remained at a maximum from 5 to 18 days, after which it declined,

reaching a steady level at about 35 days. The amount of acetylcholine given was very large and the sympathetic pathways to both eyes were intact. The records in their paper clearly suggest that the hypersensitivity declined after 18 days, but it is difficult to suppose that the method they used was reliable for detecting changes in the degree of hypersensitivity. Keil and Root suggested that the initial increase in sensitivity might be due to a loss of cholinesterase, and that the later fall in sensitivity might be due to a partial restoration of it. The present observations show that the greater part of the cholinesterase in the iris is true or specific cholinesterase ; after denervation the amount of this enzyme drops to about 40 per cent, and there is no restoration at the end of thirty-five days.

SUMMARY

When the ciliary ganglion is removed from a cat, the amount of cholinesterase, which is chiefly true or specific cholinesterase, in the iris declines. The amount in the denervated iris expressed in terms of that in the normal iris falls to about 40 per cent ; it reaches this level at about 21 days and remains there, showing no return up to 35 days. This fall in cholinesterase is the same whether the substrate used in the manometric estimation is acetylcholine or acetyl-β-methylcholine. When benzoylcholine is used as a substrate the fall is much less ; the enzyme in the iris has, however, little action on benzoylcholine.

I am indebted to Professor J. H. Burn for suggesting this work.

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A STUDY OF RESISTANCE TO ANTRYCIDE IN A STRAIN OF *TRYPANOSOMA EQUIPERDUM*

BY

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In a previous paper on the mode of action of antrycide (Ormerod, 1951a) an antrycide resistant strain of *Trypanosoma equiperdum* was used. This paper describes: (1) Methods used for preparing resistant strains and the way in which they acquired resistance. (2) Experiments designed to test the effect of drugs on infections produced by normal and resistant strains, and the mode of absorption of drugs by the two strains. (3) A change that occurred in the normal strain which was accidentally treated with a dye having mild trypanocidal activity.

The findings are discussed in relation to the hypothesis (Ormerod, 1951b) that antrycide and several other "trypanocidal" substances inhibit the growth of trypanosomes by splitting the cytoplasmic nucleoprotein into its constituent protein and ribonucleic acid, thereby preventing the interaction of these substances necessary for growth and reproduction.

MATERIALS

A normal strain of *T. equiperdum* used at the National Institute for drug standardization was employed during the course of this work; initially the cytoplasm was clear when stained with eosin-methylene blue compound stains and there was not more than one inclusion per ten trypanosomes when viewed in the living active state by phase contrast microscopy. Antrycide was provided by Messrs. Imperial Chemical (Pharmaceuticals) Ltd. as the methylsulphate. The chloride was prepared by precipitation from a solution of the methylsulphate by adding sodium chloride. "Collosol Cuprum," Crookes brand of 0.05 per cent colloidal copper, was used in normal and splenectomized mice according to the method of Hawking (1939) and von Jancsó (1934) for "reticulo-endothelial blockade."

RESULTS

The preparation of resistant strains

Resistant strain 1.—At the first attempt to form a resistant strain, one of a group of twelve mice with well established infections, previously given 0.3 ml. intravenous collosol cuprum, failed to respond to 0.01 mg./20 g. of antrycide given intraperitoneally. After 7 days 0.1 mg./20 g. of antrycide would not control this infection, which was transmitted 8 times successively to clean mice each dosed with 0.1 mg./20 g. of antrycide. The intraperitoneal dose that could then be given without controlling the infection was 0.25 mg./20 g. (the LD50 in mice by this route is 0.34 mg./20 g.). This dose, however, given on two consecutive days would cure the infection.

Rough tests using two groups of 4 mice showed no gross differences between normal and resistant strains in response to dimidium bromide and suramin.

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The strain was stored for a month in guinea-pigs, and during this time lost completely its resistance to antrycide.

Resistant strain II.—The same technique was employed to produce another resistant strain, but when after 6 transmissions in 30 days no increase in resistance had occurred, it was modified in the following way. Splenectomized mice with a well established infection were given an intravenous injection of 0.2 ml. colloidal copper followed by a subcutaneous injection of antrycide chloride. The insoluble antrycide chloride was given as a suspension of crystals, 1 mg./ml. in 10 per cent saline, so as to ensure the slowest possible absorption of the drug into the blood stream. A group of 4–6 mice was maintained in this way, infected, and treated daily ; and two fresh mice were added to the group every one or two days to replace those that had died or had been cured. At each transmission, all the mice were infected one from another. The essentials of this technique were: the maintenance of a high trypanosome count, frequent transmissions, and prolonged action of small amounts of the drug while there was as yet little resistance. When the increase in resistance seemed to warrant it, this technique was abandoned, and infected mice were dosed with antrycide methylsulphate without previous splenectomy or injection of colloidal copper.

The progress of resistance of the strain under treatment with antrycide is shown in Table I. The minimum number of possible transmissions that the resistant strain

TABLE I

PROGRESS OF RESISTANCE

Table showing the increase in resistance of the strain with the treatment outlined in the text. Fifty per cent lethal dose of antrycide methylsulphate intraperitoneally $\simeq$0.34 mg./20 g. mouse. Fifty per cent curative dose of antrycide methylsulphate intraperitoneally on the normal parent strain $\simeq$ 0.013 mg./20 g. mouse.

No. of mice added to antrycide treated group	Days from beginning of experiment	Maximum intraperitoneal dose that failed to clear the blood mg./20 g.
0	0	0.01
17	46	0.05
31	69	0.1
45	89	0.1
45	96	0.2
45	99	0.1
55	106	0.5
55	110	0.2
60	126	0.8 (in four doses)
	191	No influence on infection
	548	
	Treatment with antrycide stopped	
	594	0.05 (cleared the blood)

could have undergone is equivalent to the number of mice added to the treated group ; as each mouse was infected approximately ten times, the maximum possible number of transmissions is of the order of ten times this figure. As in resistant strain I, each increase in resistance occurred suddenly at one point in the series of transmissions, and although the technique of cross-infection between all the animals made this point difficult to define, it was clear that there was no gradual transition from susceptibility to resistance. Another point of interest in the progress of this strain from susceptibility to resistance was that loss of resistance could occur during

treatment as well as gain ; for instance, on the 106th day of the experiment trypanosomes from one mouse in the group required more than the LD50 of antrycide methylsulphate, whereas trypanosomes from another mouse in the group with a previously identical infection gave a 50 per cent cure (determined graphically from a 6-point assay) with 0.062 mg./20 g. mouse, only 2.5 times the original 50 per cent curative dose.

As is shown in Table I, the necessary curative dose 106 days after the beginning of the experiment exceeded the LD50 of the drug for mice ; from then on resistance continued to increase so that the infection was no longer influenced by repeated injections of the maximum tolerated dose of antrycide ; at this point the experiments described below were performed. Throughout their progress animals bearing the resistant strain were dosed with antrycide at each passage ; but after the experiments finished (548 days) the strain was no longer dosed and lost its resistance in a further 46 days, so that the blood of a mouse infected at the rate of one trypanosome per one-twelfth-inch oil-immersion microscopic field could be cleared by 0.05 mg./20 g. of antrycide methylsulphate.

Experiments with the fully resistant strain

Cross resistance tests.—As there was no demonstrable difference in infectivity between the normal and antrycide resistant strains, it was possible to determine the degree of resistance of the antrycide resistant strain as compared with the parent strain for any particular drug by comparing the log dose-response lines that it produced using the two strains in simultaneous experiments under identical conditions. It was assumed for this purpose that when the blood trypanosome counts of the two groups of mice were similar, any difference in response of the strains was due to their difference in resistance to the drug in question.

Mice of 18–21 g. were infected the previous day with a number of trypanosomes, approximately 200 million, adjusted to give an infection at the rate of one trypanosome per one-twelfth-inch oil-immersion field on the day of the experiment ; animals with a significantly different degree of infection were rejected from the experiment. The blood films were examined in each experiment at the point found to give the greatest difference in response between high and low doses of the drug: this was found to be at 48 hours for dimidium bromide and at 24 hours for the other drugs studied. The results with suramin were found to be best at 24 hours despite the fact that the blood of many mice still infected at 24 hours would have been cleared in 48 hours. Wet smears of approximately the same density were made from tail blood. Failure to see one trypanosome in 20 fields using a one-twelfth-inch oil-immersion objective was taken as a positive response. This relatively crude method was found to be as satisfactory as any other available for determining whether the blood of an animal was clear or infected. No account was taken of subsequent relapse or clearing of the blood, since the sole object of the experiment was to obtain satisfactory graded log dose-response lines from which the difference in resistance of the two strains could be estimated.

The results of these tests are shown in Table II. Dimidium and stilbamidine were, like antrycide, unable to clear trypanosomes from the blood of mice within the limits set by acute toxicity of these drugs, and the antrycide resistant strain may to this extent be said to be totally resistant to them. No resistance could be demonstrated to suramin: the figures suggest an increased sensitivity of the antrycide resistant strain to this drug, but do not admit of analysis for significance. The

TABLE II

Table showing the degree of cross-resistance of antrycide resistant strain II to the drugs shown in the first column. Resistance in the last column is the reciprocal of "Potency" in the usual type of drug assay. One experiment each with trypan blue and *p*-hydroxyphenylarsenoxide could be assessed statistically. With acriflavine the three experiments were combined before assessment.

Drug	Dose mg./20 g.	Normal Strain		Resistant strain		No. of deaths from drug toxicity	Resistance as compared with normal strain = 1 (fiducial limits)
		No. cleared	Total	No. cleared	Total		
Suramin:							
Exp. (i)	0.020	0	4	0	4		
	0.028	0	4	3	4		
Exp. (ii)	0.020			5	5		
	0.028	4	5	4	4		
	0.039	5	5				
Exp. (iii)	0.020	0	5	0	5		
	0.025	0	6	1	6		
	0.030	1	6	4	6		
	0.035	1	5	2	5		
Trypan blue:							
Exp. (i)	0.7	0	10	1	10		
	0.8	1	10	7	10		0.877
	0.9	4	10	6	10		(0.76–1.08)
	1.0	7	10	7	10		
Exp. (ii)	0.5	0	6	0	6		
	0.6	0	6	3	6		
	0.7	0	6	3	6		
	0.8	0	6	3	6		
	0.9	2	6	4	4		
p-Hydroxyphenyl-arsenoxide:							
Exp. (i)	0.015	0	5	0	5		
	0.017	2	5	2	5		1.09
	0.020	4	5	2	5		(0.85–1.31)
	0.0225	5	5	4	5		
Exp. (ii)	0.01	0	6	0	6		
	0.02	4	6	5	6		
	0.03	6	6	6	6		
Acriflavine:							
Exp. (i)	0.15	0	10				
	0.19	2	10				
	0.24	2	9				
	0.25			0	10		
	0.31			3	9		
	0.39			6	10		
Exp. (ii)	0.19	3	10				
	0.24	9	10				
	0.25			3	10		1.31
	0.30	10	10				(1.19–1.44)
	0.31			8	10		
	0.39			10	10		
Exp. (iii)	0.19	0	9				
	0.24	2	8				
	0.25			0	9		
	0.30	6	8				
	0.31			4	9		
	0.39			10	10		

TABLE II—*continued*

Drug	Dose mg./20 g.	Normal strain		Resistant strain		No. of deaths from drug toxicity	Resistance as compared with normal strain=1 (fiducial limits)
		No. cleared	Total	No. cleared	Total		
Dimidium	0.2	0	8			0	
	0.4	3	5			0	
	0.5	1	5	0	5	0	
	0.7	2	5			0	
	0.8	3	7	0	5	0	>2.3*
	1.0	4	5			0	
	1.25			0	5	1	
	1.28			0	4	1	
	1.6			0	5	2	
	2.05			0	10	7	
Stilbamidine ..	0.48	4	10			0	
	0.57	8	10			0	
	0.69	7	10			0	>3.2*
	1.60			0	10	0	
	2.40			0	10	5	
	3.60			0	10	10	
Antrycide	0.004	1	10				
	0.008	4	10				
	0.016	5	10				
	0.032	9	10				
	0.27				18	3	>26.0*
	0.32				18	10	

* LD50/ED50 for normal strain.

figures for trypan blue in the same way suggest an increased sensitivity of the " resistant " strain, but this is not significant. There is no significant difference in the responses of the two strains to *p*-hydroxyphenylarsenoxide. An experiment with tryparsamide was not completed because of the change in the normal strain (described below). A small but statistically significant difference in the response of the two strains to acriflavine was shown, and represented a 1.3-fold increase in resistance.

The absorption of drugs visualized by fluorescence microscopy.—A study was made of the fluorescence of normal and resistant strains after absorption of antrycide. An Osira lamp and a Wrattan 18A filter with transmission maximum in the 3650A region were used. The typical appearance in the normal strain was the same as previously described (Ormerod, 1951a). (The highly fluorescent spot described previously was now identified as the kinetoplast and not the basal vacuole as I had then supposed.) One hour after a mouse had been treated with a large dose of antrycide, 0.2 mg./20 g., the kinetoplast in the trypanosomes of both strains became fluorescent with white light, and the cell body uniformly fluorescent with blue light. After about four hours blue granules appeared in the cytoplasm, most numerous in the resistant trypanosomes. After 24 hours the kinetoplast was still fluorescent but not the cell body of the antrycide resistant trypanosomes, in which only the kinetoplast could be seen ; but in the normal trypanosomes the kinetoplast and numerous granules were fluorescent with a white light.

When 2-hydroxystilbamidine was injected into infected mice the effect on the two strains was most striking. At three hours after injection of 0.5 mg./20 g. of 2-hydroxystilbamidine (the time at which there was the greatest differentiation between the two strains) trypanosomes of the normal strain showed a bright yellow kinetoplast and one or more yellow granules of the same size and intensity in the cytoplasm. Trypanosomes of the resistant strain showed the same bright yellow kinetoplast, but the granules appeared smaller and with a blue fluorescence.

Plates I and II show trypanosomes of the normal and the resistant strains treated with 2-hydroxystilbamidine. In both strains the kinetoplast fluoresces orange ; in the resistant strain the small granules (which are already present in the untreated organism) have absorbed the drug and produce a blue fluorescence. On the other hand, the large granules which appear in the normal strain are produced by the drug which gives a brilliant orange fluorescence fading little on exposure to ultra-violet light. Similar changes occurred in trypanosomes treated with stilbamidine giving the same distinction between normal and resistant strains.

PLATE I.—Normal *T. equiperdum* in a dry unstained blood smear taken 3 hours after 0.5 mg./20 g. 2-hydroxystilbamidine. Both kinetoplast and inclusions produced a brilliant orange-yellow fluorescence when irradiated with ultra-violet light in the 3650A region.

PLATE II.—Antrycide resistant *T. equiperdum* in a dry unstained blood smear taken 3 hours after 0.5 mg./20 g. of 2-hydroxystilbamidine. The kinetoplast produced an orange-yellow and the inclusions a blue fluorescence when irradiated with ultra-violet light in the 3650A region.

The production of granules in a strain that previously produced trypanosomes with agranular cytoplasm

When the antrycide resistant strain had been fully formed, it was noticed that the cytoplasm contained granules whereas that of the normal parent strain was relatively clear when examined by phase-contrast microscopy. These granules could not be stained with eosin-methylene blue compound stains but were otherwise similar to the inclusions produced by drugs. However, an accident occurred in the laboratory which will be described in detail, as it provides an example of the formation of granules while the strain was under microscopic control. On March 19, 1951, the normal strain was examined by phase contrast and inclusions were seen at a rate not exceeding one per ten trypanosomes; on April 3, when the strain was re-examined the cytoplasm of each trypanosome was found crammed with granules of varying sizes. These granules were highly refractile and appeared as purplish spots under ordinary light microscopy, and showed a yellow fluorescence under ultra-violet. They did not stain with eosin-methylene blue compound stains. It was then noticed that the mice were excreting pink urine, and on inquiry it was discovered that since weaning they had been fed on a diet containing the dye, Rhodamine B

(Edicol Rose B) Colour Index No. 749, in a concentration of 1:18,000. A large dose of this dye, 0.5 mg./20 g., was found to clear the blood of trypanosomes, but relapse occurred within two days. It is uncertain for how long the rats and mice in the Institute were receiving this diet, for although this diet was removed as soon as the effect was noted it is likely that traces remained in the bodies of animals for at least a month.

In two months' time when all animals were free from dye, never having received the diet that contained it, the granules still persisted in the trypanosomes ; they were, however, no longer coloured or fluorescent, but as numerous and as refractile as ever. A dose of 1.0 mg./20 g. of Rhodamine B (the maximum tolerated dose for mice) would no longer clear the blood, and in several experiments a slight resistance to antrycide could be detected, but its degree could not be evaluated because it seems to have been unstable, and because there was now no *normal* parent strain with which to compare it. Thirteen months later these granules were still present although smaller and in diminished numbers.

Discussion

The use of colloidal copper

The rationale for using colloidal copper in preparing drug resistant strains is based on the theories of von Jancsó and von Jancsó (1935), who found that a dose of colloidal copper antagonized the therapeutic action of suramin on trypanosomes and caused the appearance after suramin of abnormal multinuclear forms. They believed that the action of suramin was to coat the trypanosome in the manner of an " opsonin " so that it could be seized by reticulo-endothelial cells: when the reticulo-endothelial system was poisoned by a dose of colloidal copper the abnormal forms would then be able to survive, and among the trypanosomes that would be spared in this way would be the partially resistant forms. Hawking (1939) has used this technique successfully in preparing a suramin resistant strain, so it seemed reasonable to apply it to the formation of an antrycide resistant strain, all the more since preliminary experiment had shown that " collosol cuprum " had a marked inhibitory effect on the therapeutic action of antrycide.

But soon it became clear that some different mechanism was in operation ; the reasons for this were as follows:

(1) Treatment with " collosol cuprum " delayed the appearance of basophilic inclusion bodies which usually occur in trypanosomes within twenty-four hours after an effective dose of antrycide. The inclusion bodies did not occur at all when the therapeutic effect of antrycide was completely inhibited.

(2) Previous intraperitoneal administration of " collosol cuprum " diminished slightly the toxicity of intraperitoneal antrycide methylsulphate.

(3) Antrycide forms a precipitate *in vitro* with " collosol cuprum."

(4) Multinuclear forms similar to those referred to by the von Jancsós were produced (Ormerod, 1951a) in *T. rhodesiense* (without treatment with colloidal copper) by a fifth of the dose of antrycide needed to clear the blood of trypanosomes.

These experiments were not pressed beyond the point where it became likely that a process other than blockade of the reticulo-endothelial system was influencing the increase in antrycide resistance, since although they throw doubt on the conclusions of the von Jancsós, it seemed fruitless to repeat their actual experiments, as they did not indicate the exact nature of the colloidal copper that they used or its method of preparation. In " collosol cuprum " the colloidal copper is stabilized with peptone and cresol ; other

stabilizing agents can be used such as egg albumin, gum arabic, or agar, and as some of these might combine with suramin and others not, it is impossible to say whether the therapeutic inhibition that the von Jancsós observed was actually produced by reticulo-endothelial blockade or by fixation of the drug in the way that " collosol cuprum " seems to be able to fix antrycide *in vivo*.

The benefits of using colloidal copper in the production of an antrycide resistant strain—if indeed there are benefits—probably consist in keeping the organisms subjected to a low concentration of the drug for long periods.

The mechanism of antrycide resistance

These experiments, as far as they have been pursued, have shown a distinction in the reactions of the antrycide resistant strain between acidic and basic drugs. No resistance to suramin, trypan blue, or *p*-hydroxyphenylarsenoxide was demonstrable, but to the basic drugs dimidium and stilbamidine there was well-marked resistance, and there was slight resistance to acriflavine.

Most workers who have studied the relative absorption of drugs by normal and resistant strains of trypanosomes agree that the resistant strains show a diminished absorption of the drug ; much of this work is epitomized by that of Hawking (1934, 1937, and 1944), who, using biological and chemical methods of estimation, was able to show that the arsenicals, acriflavine, and stilbamidine were absorbed in considerable quantities by normal trypanosomes, but in smaller amounts by resistant trypanosomes. Suramin was absorbed by the normal strain in such small amounts that he was unable to detect differences in absorption between normal and resistant strains (Hawking, 1939). In this investigation I have not been able to detect such differences in the absorption of antrycide by the two strains beyond the different appearance that the two strains show on examination by fluorescence microscopy, and these cannot be interpreted quantitatively because of the colour and intensity differences. Qualitatively, however, it is clear that antrycide, stilbamidine, and 2-hydroxystilbamidine are able to penetrate the trypanosome cell of the resistant as well as the normal strain, but the normal cell seems to retain the drug in its substance, while it disappears from the resistant strain and does not affect its growth and metabolism. A strong suggestion as to the meaning of the colour differences between the two strains comes from the work of Oster (1951), who showed that a small shift occurred in the wavelength of acriflavine on forming a complex salt with nucleic acid. More spectacular use of the same phenomenon was made by Snapper and others (1951), who showed an even more striking shift in the wavelength of 2-hydroxystilbamidine on combination with ribonucleic acid: the hydrochloride of 2-hydroxystilbamidine produces a faint blue fluorescence, but in combination with nucleic acid it changes to a brilliant orange yellow. Snapper and his co-workers also showed that the brilliant orange yellow fluorescence was given from cell structures known to be rich in ribonucleic acid when the cell had absorbed the drug. While this orange-yellow is certainly not specific for nucleic acids—it is produced by combination of 2-hydroxystilbamidine with other polybasic acids such as heparin and to some extent with ethionic acid—the observations of bright orange-yellow bodies in the normal strain of trypanosomes indicate that the drug is fixed by acidic structures in the cell, and evidence has already been put forward (Ormerod, 1951b) to suggest that such structures are composed largely of protein and ribonucleic acid. The blue fluorescence of the bodies in the cytoplasm of the resistant strain shows that in some way

the drug is prevented from combining with these structures. In this connection it is interesting that Schueler (1947) has suggested a shift in the isoelectric point of cytoplasmic protein of resistant trypanosomes, since he observed differences in staining capacity of drug-resistant trypanosomes at increased hydrogen ion concentrations.

In spite of this clear-cut mechanism it should not be assumed that this is the only way that a trypanosome can acquire drug resistance. The sudden increases in resistance that were observed may represent the acquisition of other mechanisms of drug resistance, and the fact that the resistance of the strain for antrycide is greater than for other drugs may suggest other mechanisms more specific for antrycide.

It is tempting to suppose that the inclusion bodies found by phase contrast examination of the antrycide resistant strain correspond to those produced by rhodamine and are an essential part of the mechanism of drug resistance ; it must, however, be emphasized that these changes were found by accident, and in the one instance the way in which the inclusions developed was not observed, and in the other the conditions under which they developed were not under control.

Conclusion

As a final conclusion it can be stated that nothing in this work has been shown to be inconsistent with the working hypothesis (Ormerod, 1951b) that antrycide and several other drugs inhibit the growth of trypanosomes by splitting the cytoplasmic ribonucleoprotein into its constituent protein and ribonucleic acid. To this group of drugs it is now possible to add stilbamidine and 2-hydroxystilbamidine. True, they behave differently in other ways, as Hawking (1944) has shown that stilbamidine can be trypanocidal *in vitro,* and the inclusion bodies which it produces in less than half an hour's treatment are not basophilic and can only be demonstrated by the fluorescence in the dry smear or by phase contrast in the living organism. A further addition to the hypothesis concerns a mechanism of resistance that has been demonstrated in this work: whereas the nucleoprotein in the cytoplasm of the normal trypanosome is reactive and can be split by drugs, the nucleoprotein of the antrycide resistant organism is for some reason unreactive to antrycide and other basic drugs so that they are able to enter and leave the cytoplasm without damaging the cell or inhibiting its growth.

Summary

1. Two antrycide resistant strains of *T. equiperdum* were prepared using "collosol cuprum." Evidence is given in brief to suggest that the action of "collosol cuprum" is to fix the drug so that the organism is subjected only to small amounts.

2. The resistant strains were unstable and had to be maintained with constant doses of antrycide.

3. Resistant strain II showed cross resistance that was complete—within the limits set by the toxicity of the drug for mice—to dimidium and stilbamidine ; partial resistance to acriflavine was observed, but no resistance to suramin, trypan blue, and *p*-hydroxyphenylarsenoxide.

4. Inclusion bodies were observed in the cytoplasm of the normal strain of *T. equiperdum* that had previously been free from granules but had been treated

accidentally with Rhodamine B. These inclusion bodies could not be stained with eosin-methylene blue compound stains but were demonstrated by phase-contrast microscopy. At first they contained the dye but persisted for many months after its withdrawal in the descendants of the affected trypanosomes.

5. Inclusion bodies with similar properties were observed in the fully developed antrycide resistant strain.

6. A striking differentiation between the normal and resistant strains is produced by treatment with 2-hydroxystilbamidine. In trypanosomes of the normal strain inclusion bodies appear with bright orange fluorescence (these are produced by the drug) and in the resistant strain many blue granules (present in the organism before exhibition of the drug) are shown. Similar but less striking changes occur with antrycide and stilbamidine.

7. A mechanism of drug resistance has been demonstrated in which the cytoplasmic nucleoprotein, which is normally split by basic tryanocidal drugs into protein and a combination of the drug with ribonucleic acid, becomes unreactive so that these drugs can no longer combine with it. They therefore leave the cell when the blood concentration falls, since they are not bound to the cell structure. This is not likely to be the only mechanism whereby a trypanosome can acquire drug resistance, but in this instance it is probably the most important.

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CHOLINESTERASE LEVELS IN THE NERVOUS SYSTEM IN TRI-*ORTHO*-CRESYL PHOSPHATE POISONING

BY

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In 1930 Smith and Elvove published a report on a localized outbreak of motor neuritis in the United States. It was shown that the paralysis followed the consumption of a drink known as " ginger jake," and was apparently due to the presence of tri-*ortho*-cresyl phosphate (TOCP) in the fluid extract of ginger used in the preparation of the drink (Smith, Elvove, and Frazier, 1930). Since then cases of paralysis following accidental ingestion of this compound have been described many times (among others, Ter Braak, 1931 ; Germon, 1932 ; Sampson, 1938 ; Aring, 1942 ; Hunter, Perry, and Evans, 1944 ; Hotston, 1946).

The syndrome of TOCP poisoning is usually described as a peripheral neuritis, although it is clear that cord changes are also present. Motor function is severely affected, and the paralysis is more marked in the feet and legs than in the arms. The distal muscles of the limbs are more affected than those nearer the trunk. Sensation seems to be largely unaffected. Complete recovery may ensue, but in some patients late signs may appear of damage to the pyramidal tracts, and the picture then has been described as being very like that seen in amyotrophic lateral sclerosis (Aring, 1942). On histological examination, demyelination is found both in the peripheral nerves and in the white matter of the spinal cord (Smith and Lillie, 1931 : Aring, 1942). Paralysis and demyelination can also be regularly and easily produced by this compound in experimental animals, particularly the hen (Smith, Elvove, and Frazier, 1930 ; Smith, Engel, and Stohlman, 1932 ; Smith and Lillie, 1931).

The study of the immediate cause of demyelinating diseases in man is difficult. It was felt, therefore, that the experimental production of a comparable condition by this compound in animals would be of value in that it would provide an opportunity to gain more insight into the changes in the nervous system that may accompany demyelination. In this instance we should be concerned with demyelination resulting from the action of a single, simple chemical substance, and the condition should therefore lend itself to the study of any biochemical and enzymic changes in the nervous system which might be associated with the histological lesion.

As far as we are aware, the only biochemical effect of trio-*o*-cresyl phosphate which has been described is the inhibition of cholinesterase and tributyrinase activity (Bloch, 1941 ; Hottinger and Bloch, 1943 ; Mendel and Rudney, 1944). Bloch suggested that inhibition of the cholinesterase at the motor end-plates might be the cause of the paralysis. On such a view, however, it would be difficult to account for the

histological changes observed and also the delay of about 14 days before the onset of symptoms.

It is now known that two different cholinesterases are present in the body: the true cholinesterase present at synapses and at the motor end-plates, and the so-called " pseudo-cholinesterase " present in serum and in a number of other tissues, including the nervous system. The true cholinesterase is usually regarded as the enzyme which hydrolyses acetylcholine liberated at nerve-endings, whereas the function of the pseudo-cholinesterase is unknown. Bloch's view, therefore, assumes an inhibition of the true cholinesterase, whereas TOCP appears to be an inhibitor of pseudo-cholinesterase (Mendel and Rudney, 1944; Earl and Thompson, 1952). We have shown that the true cholinesterase present in human muscle and nervous tissue is not significantly inhibited *in vitro* by TOCP. On the other hand, we found a marked and selective inhibition of the pseudo-cholinesterase in human nervous tissue and serum; a similar selective action was also found in the tissues of the hen. We suggested that inhibition of the pseudo-cholinesterase in the nervous system might result in some disturbance of the normal maintenance of the myelin sheaths of nerve fibres, and so cause demyelination. In this connection a recent report (Bidstrup and Hunter, 1952) is of interest in which three cases of poisoning by a new anti-cholinesterase insecticide were described; two of the patients developed a late flaccid paralysis resembling that which follows poisoning by TOCP.

In the work to be described we therefore set out to study the cholinesterase levels in the nervous system of hens poisoned and paralysed by TOCP, in order to determine whether in the poisoned animal as well as under our earlier *in vitro* conditions the same selective inhibition of the pseudo-cholinesterase is found.

Materials and Methods

Hens weighing between 1,400 and 3,000 g. were used in this work. They were poisoned by a single dose of TOCP (1 ml./kg. body wt.), administered by mouth with a 2 ml. graduated pipette.

Blood was taken by venepuncture from the wing vein, and was immediately heparinized. Animals were killed by decapitation at varying intervals (up to 21 days) after intoxication. The whole brain and spinal cord were removed immediately after death, dissected free from membranes, and homogenized in 0.025 M–$NaHCO_3$ to give 1 in 15 (w/v) dilutions.

Cholinesterase determinations were carried out manometrically in the Warburg apparatus at 38°. CO_2 evolutions were measured for periods up to 1 hr. Results for plasma are expressed as μl. CO_2/ml./min. and for tissues as μl. CO_2/g. (wet wt.)/hr.

Tri-*o*-cresyl phosphate, prepared from pure *o*-cresol, was obtained from Geigy Pharmaceuticals, Ltd.

Substrates.—In order to differentiate between the true and pseudo-cholinesterases that may be present in a given tissue preparation we have used " selective " substrates. For the true cholinesterase we have used 0.03 M-acetyl-β-methylcholine chloride (Savory & Moore, Ltd.), which was shown by Mendel, Mundell, and Rudney (1943) to be readily hydrolysed by this enzyme, though attacked only very slowly by the pseudo-cholinesterase of most animal species. In the course of this work, however, we discovered that this substrate is hydrolysed by the pseudo-cholinesterase of chicken plasma, rather more rapidly than by the corresponding enzyme in most other species. In the hen, therefore, acetyl-β-methylcholine is an appropriate substrate for the true cholinesterase when this

enzyme is present in the tissue preparation in much larger amounts than the pseudo-cholinesterase (as in brain), although it is not an ideal selective substrate when much of the latter enzyme is present. This point will be referred to later.

Butyrylcholine, on the other hand, is hydrolysed rapidly by the pseudo-cholinesterase (Stedman, Stedman and Easson, 1932), but only very slowly by the true cholinesterase (Nachmansohn and Rothenberg, 1945). For the estimation of pseudo-cholinesterase activity we have therefore used 0.03 M-butyrylcholine chloride (B.D.H., Ltd.), or butyryl-choline perchlorate, prepared by Dr. G. R. Webster in this laboratory. Butyrylcholine chloride was employed in the earlier experiments, but the perchlorate was substituted in the later ones, since it was found to be a more satisfactory substrate, in that it is much less deliquescent than the chloride. Both salts were found to be hydrolysed at the same rates by human serum cholinesterase.

RESULTS

Estimations of cholinesterase levels have been made on 24 poisoned birds. For about ten days after poisoning the birds remained apparently normal. At any time from the tenth to the fourteenth day the birds showed the first definite signs of paralysis; they appeared quite normal in the cage, but when let out they walked unsteadily. At a later stage, perhaps the next day, the birds would be seen sitting on their heels in the cage; when released they walked unsteadily at first, and after a few minutes they were unable to lift their heels from the ground. In a few days the legs became completely useless and the birds sat with their legs outstretched, unable to move. In some birds the claws were flexed and in others they were extended. Birds in the most severe stages showed some weakness of the wings, and some of the most severely affected showed slow respiration with gaping of the beak. Apart from the paralysis, however, they seemed healthy. They held their heads high and their combs were bright, and except in advanced stages of paralysis they ate well.

A summary of the signs shown by the poisoned birds at the time of killing is given in Table I. Only two birds in the series died, one two days after poisoning,

TABLE I

SIGNS SHOWN BY HENS POISONED WITH TOCP (1 ML./KG. *per os*) AT TIME OF KILLING

(All birds killed before the 8th day appeared normal)

Days after poisoning	Symptoms
8	Slight leg weakness after exercise.
8	Nil.
10	Nil.
12	Nil.
12	Slight leg weakness. Walks on heels.
14	Unable to stand. Walks on heels.
14	Unable to stand.
14	Unable to stand. Sits with legs outstretched.
15	,, ,, ,, ,, ,, ,, ,,
15	Unable to stand.
17	Unable to stand. Sits with legs outstretched.
19	,, ,, ,, ,, ,, ,, Wings weak.
19	,, ,, ,, Wings weak. Gaping.
20	Slight leg weakness. Unsteady gait.
21	Unable to stand. Ataxic.

and the other a few minutes after a venepuncture on the 14th day. The causes of death were undiscovered.

Plasma cholinesterase level.—In view of the sensitivity *in vitro* of the pseudo-cholinesterase of the plasma to inhibition by TOCP, estimations of this enzyme were made on blood samples from 26 poisoned birds, in some serially. Estimations were also carried out on samples withdrawn from all birds immediately before poisoning. By this means it was hoped to obtain evidence of adequate absorption of the orally administered poison. Estimations were also made on plasma samples from a further 20 normal birds. Repeated estimations on the plasma level of a given normal bird showed no great variation. Although the range of the level of plasma cholinesterase (using butyrylcholine as substrate) in different birds is somewhat large (from 10.8–30.0 μl./ml./min.; mean 20.2 μl./ml./min.), there was a sharp fall in the level (Fig. 1), as

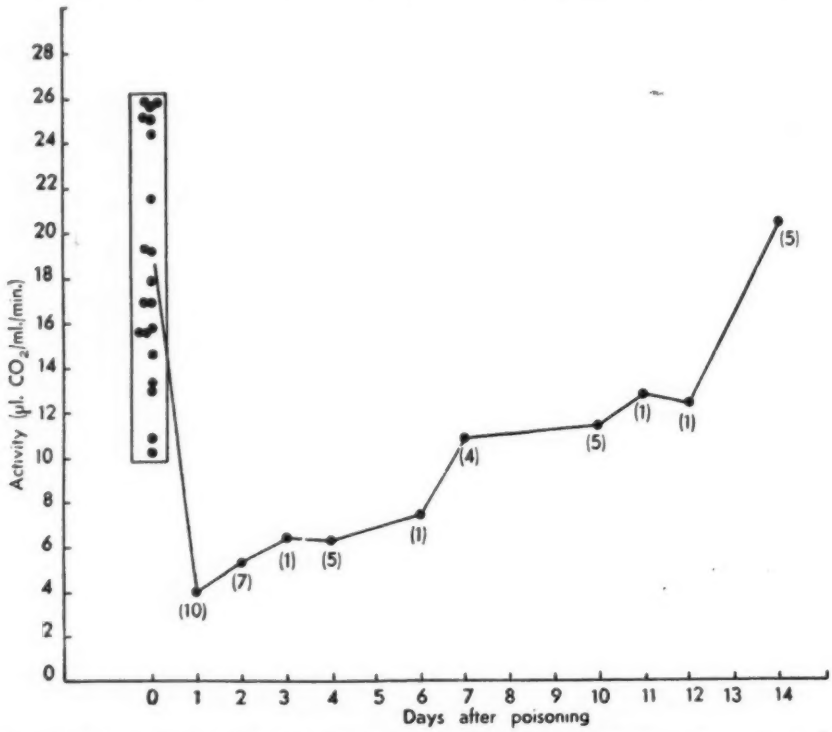

FIG. 1.—Plasma cholinesterase levels in hens after poisoning with TOCP (1 ml./kg. *per os*). (Numbers in brackets denote number of birds used.)

early as 3–4 hr. after administration of the TOCP, to values varying from about 1.6–8.0 μl./ml./min. The plasma cholinesterase activity remained at a low level for 4–6 days, after which it rose slowly, reaching the normal range by about 14 days. It is of interest that TOCP, which is a rather viscous oil, is absorbed sufficiently rapidly from the hen's gastro-intestinal tract to show approximately maximum inhibition of the plasma enzyme by 3–4 hr.

Cholinesterase levels in the brain.—The levels of true and pseudo-cholinesterase were estimated in the brains of 23 animals killed at varying times after poisoning

TABLE II

MEAN CHOLINESTERASE (CHE) VALUES IN NERVOUS SYSTEM OF NORMAL HENS AND OF HENS POISONED WITH TOCP (1 ML./KG. *per os*).

Activity expressed as $\mu l.CO_2/g./hr.$ (Numbers in brackets denote number of birds studied.)

	Days after poisoning	Brain		Spinal Cord	
		Pseudo ChE	True ChE	Pseudo ChE	True ChE
Normal ..	—	2,490 (13)	12,650 (14)	1,815 (12)	2,450 (11)
Poisoned ..	1	728 (2)	11,220 (2)	455 (2)	1,880 (2)
	2	860 (3)	11,790 (3)	305 (2)	1,485 (2)
	3	846 (1)	11,010 (1)	511 (1)	2,256 (1)
	4	972 (1)	11,720 (1)	207 (1)	1,533 (1)
	5	447 (1)	12,600 (1)	—	—
	6	—	—	285 (1)	1,340 (1)
	8	771 (2)	10,900 (2)	375 (2)	1,260 (2)
	10	690 (1)	10,260 (1)	640 (1)	2,100 (1)
	12	934 (2)	12,320 (2)	556 (2)	1,720 (2)
	14	1,201 (3)	10,940 (3)	775 (3)	1,975 (3)
	15	1,295 (2)	11,660 (2)	765 (2)	2,425 (2)
	17	1,527 (1)	13,830 (1)	981 (1)	2,280 (1)
	19	1,487 (2)	12,170 (2)	505 (2)	1,675 (2)
	20	1,323 (1)	10,300 (1)	1,005 (1)	2,100 (1)
	21	1,770 (1)	14,880 (1)	560 (1)	2,001 (1)

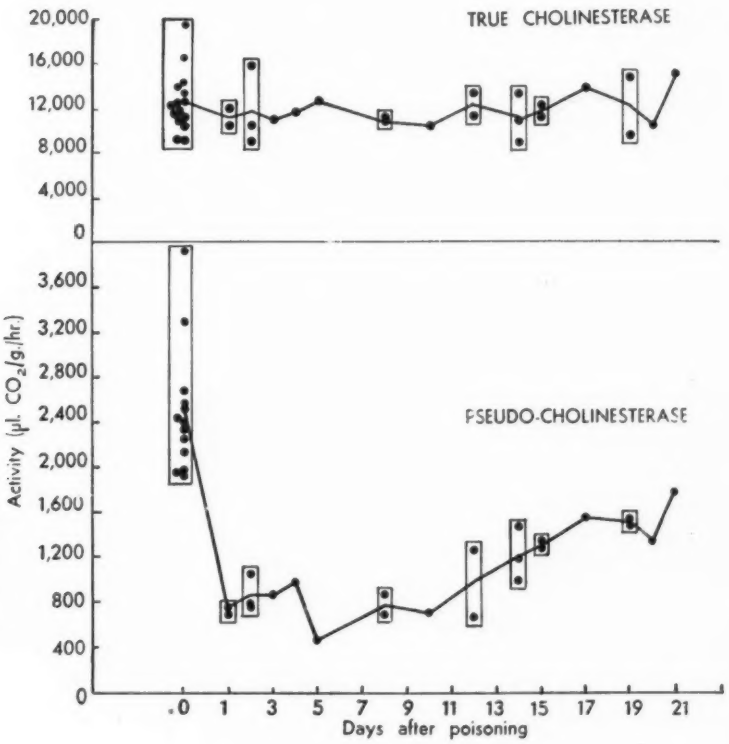

FIG. 2.—Cholinesterase levels in brain of hens after poisoning with TOCP (1 ml./kg. *per os*).

(Table II). In this tissue also the pseudo-cholinesterase activity was markedly reduced one day after intoxication, as compared with the values found in 13 normal birds (Fig. 2) ; the mean normal level of 2,490 μl./g./hr. (range 1,915–3,930 μl./g./hr.) being reduced to values between 600 and 800 μl./g./hr. It remained at this low value for about 10 days, after which a slow return towards the normal took place. The activity of the true cholinesterase, on the other hand, was unchanged throughout the period of intoxication, all the values falling within the normal range.

Cholinesterase levels in the spinal cord.—Enzyme estimations were carried out on spinal cord preparations from 22 of the poisoned animals killed for estimation of the brain levels. As in the brain, the pseudo-cholinesterase of the spinal cord was found to be profoundly diminished one day after poisoning. The mean normal value of 1,815 μl./g./hr. (range 1,245–2,260 μl./g./hr.) was reduced to about 200–700 μl./g./hr., amounting to a 75–90 per cent inhibition of activity (Fig. 3). This

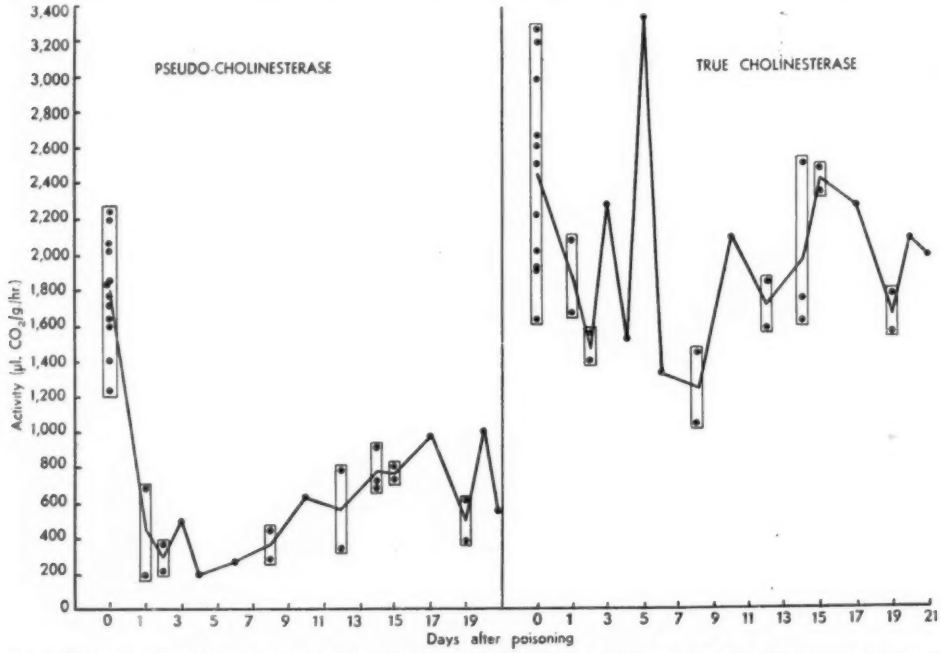

FIG. 3.—Cholinesterase levels in spinal cord of hens after poisoning with TOCP (1 ml./kg. *per os*)

low level was maintained for about 10 days (Table II), as with brain, and although there was a tendency for it to rise later, low values were still found as late as 21 days after poisoning.

The true cholinesterase values in the spinal cord of the poisoned animals showed a slight fall as compared with the normal mean, although most of the estimations fell just within the lower limit of the normal range. This slight fall in the true cholinesterase values in the spinal cord, as compared with the unchanged values in the brain, is probably not due to actual inhibition of this enzyme but to the presence of the relatively large amount of pseudo-cholinesterase in the spinal cord ; as we have earlier shown (Earl and Thompson, 1952) the pseudo-cholinesterase in the hen is able to hydrolyse, to a certain extent, the acetyl-β-methylcholine which we were

using as a substrate for measuring true cholinesterase activity. Inhibition of the pseudo-cholinesterase is therefore apparently responsible for the fall in the cholinesterase activity.

Discussion

The main fact that emerges from these experiments is that in hens poisoned with TOCP the pseudo-cholinesterase activity of the plasma and of the brain and spinal cord, removed at intervals after the administration of the poison, is appreciably lowered. The true cholinesterase activity of these tissues, on the other hand, remains almost unaffected. In these respects, therefore, TOCP acting in the intact, living organism behaves as it does under *in vitro* conditions (Earl and Thompson, 1952). The depression of pseudo-cholinesterase activity comes on rapidly, a profound fall being found in the serum three hours after administration of the drug, and in the central nervous system after one day (the shortest time studied). In the brain and spinal cord this fall persists for about ten days at least, after which the activity gradually rises ; in the serum the restoration of pseudo-cholinesterase activity appears to be somewhat more rapid.

It is known that in man the plasma cholinesterase level is lowered in states of malnutrition (Hutchinson, McCance, and Widdowson, 1951). In the poisoned birds, however, loss of weight did not occur until after some days ; the appetite, as already pointed out, failed noticeably only in the advanced stages of paralysis. Since the changes in cholinesterase levels in both the serum and the central nervous system were present and maximal one day after poisoning, it is clear that this was not due to any malnutrition arising from other actions of the poison, or from the paralysis preventing the birds from feeding. From our earlier *in vitro* results, therefore, we are of the opinion that these *in vivo* findings represent a direct toxic effect of the TOCP on the enzyme in these tissues.

We had earlier shown that the true cholinesterase present in human and rabbit skeletal muscle was not affected by concentrations of TOCP that produce an almost complete inhibition of the pseudo-cholinesterase in the central nervous system. Owing to the low level of cholinesterase activity in the skeletal muscle of the hen we have not been able to obtain reliable estimates of the activity of this enzyme in this tissue in poisoned birds. Our finding, however, that the true cholinesterase is unaffected in the brains of poisoned animals, together with the *in vitro* insensitivity to TOCP of this enzyme in skeletal muscle, is not in agreement with the view put forward by Bloch (1941) and revived by Koelle and Gilman (1949) that the paralysis is due to inhibition of this enzyme at the motor end-plates, resulting in an excessive local accumulation of acetylcholine.

From the earlier work in man and animals it seems generally agreed that the primary change in TOCP poisoning is a demyelination. Smith and Lillie (1931) have even described the compound as a " specific myelin poison." Professor G. Payling Wright has kindly undertaken histological studies on material from poisoned birds of the present series and has confirmed the presence of demyelination both in peripheral nerves and in certain of the tracts in the cord ; this work is still in progress and will be described in a later paper.

Tri-*o*-cresyl phosphate is chemically an unreactive compound. Apart from its inhibitory action on pseudo-cholinesterase and its less powerful effect on tributy-

rinase (Hottinger and Bloch, 1943 ; Mendel and Rudney, 1944 ; Earl and Thompson, 1952) it is not known to cause any other biochemical derangement. Experiments are in progress to study its effects on other enzyme systems, and from this work it already seems that the oxidation of glucose and of pyruvate by chicken brain is unaffected by the presence of TOCP *in vitro* and that the brains of poisoned animals oxidize these substrates at the normal rate.

The rapid onset of the fall in pseudo-cholinesterase activity in the poisoned birds, together with the normal behaviour of the birds for about 14 days after poisoning, suggest very strongly that this biochemical effect precedes any structural change in the nervous system, and is not the result of the demyelination. It is possible, indeed, that inhibition of the pseudo-cholinesterase may be responsible for the demyelination.

Many different inhibitors of both pseudo and true cholinesterases, some reversible and others irreversible, are now known. If therefore inhibition of the pseudo-cholinesterase in the nervous system is to be regarded as causing demyelination, it might be expected that some of these other anti-cholinesterase drugs should also cause the same change. We have referred to the two patients reported by Bidstrup and Hunter (1952) who developed a late flaccid paralysis as a result of poisoning by an anti-cholinesterase insecticide, bis-mono*iso*propylaminofluorophosphine oxide, and Barnes and Denz (private communication) have shown that it is possible to produce demyelination and paralysis in chickens both by this compound and by di*iso*propylfluorophosphonate (DFP), the histological and clinical conditions closely resembling those produced by TOCP. Both these compounds inhibit the pseudo and the true cholinesterases and in toxic doses cause initial symptoms of acetylcholine accumulation. In Bidstrup and Hunter's patients these early symptoms were treated successfully with large doses of atropine ; in their animal experiments Barnes and Denz also injected atropine in order to prevent undue activity of accumulated acetylcholine and so allow the hens to survive and show the late development of demyelination and paralysis.

The effects of toxic and even lethal doses of DFP in animals have often been described in the past, and a few cases of poisoning in man by cholinesterase inhibitors have been reported, but the development of a late paralysis of this type has not heretofore been mentioned. This may have been because the poisoned animals died from the effects of the undestroyed acetylcholine in the acute stages of intoxication, before sufficient time had elapsed for these late effects to appear. In survivors, the dose of the poison may have been too small, or its action too short-lived, to cause these late changes.

It seems, therefore, that in addition to TOCP these other two inhibitors of pseudo-cholinesterase can also produce demyelination provided that the animal is tided over the initial cholinergic effects with atropine.

The presence of pseudo-cholinesterase in the nervous system has been demonstrated in a number of different species (Boell, 1945 ; Sawyer, 1946 ; Augustinsson, 1948 ; Zeller, 1949 ; Ord and Thompson, 1950 ; Burgen and Chipman, 1951), but its detailed study has been somewhat neglected in favour of the more abundant true cholinesterase. Ord and Thompson (1952) however, showed that it is present in relatively large amounts in the white matter of human brain (and of the brain in a number of different animal species), and suggested that it might play some part in the

maintenance of the myelin sheath rather than directly with the conducting properties of the axis cylinders. More recently, together with Dr. G. R. Webster, we have shown that human peripheral nerve also contains significant amounts of pseudo-cholinesterase.

There is, unfortunately, little evidence concerning the physiological function of the pseudo-cholinesterase inside the body. Hawkins and Gunter (1946) have claimed that *in vivo* inhibition of this enzyme in the blood and tissues does not elicit symptoms indicative of the accumulation of acetylcholine, and our own observations with TOCP support this conclusion. There is, therefore, no clear reason for assuming that the pseudo-cholinesterase is concerned with the inactivation of acetylcholine. In view of the widespread distribution of this enzyme in the body it is conceivable that it may be generally concerned with some unknown aspect of metabolism, which is of particular importance in the formation or turnover of myelin; a transient inhibition of this enzymic process might cause no serious upset in the slow but continuous breakdown and formation of myelin, whereas a prolonged inhibition, such as we have shown to be produced by TOCP, might after some time cause defects in myelination to become apparent.

There are, of course, difficulties in the way of accepting the simple view that inhibition of the pseudo-cholinesterase in nervous tissue can result in demyelination. Our findings indicate, however, that the demyelination produced by TOCP is associated with an inhibition of this enzyme in the nervous system, and that this inhibition is an immediate effect which persists for about ten days and therefore precedes the structural and physiological damage.

SUMMARY

1. Hens have been poisoned by the oral administration of a single dose of tri-*ortho*-cresyl phosphate (1 ml./kg. body wt.). Under our conditions this dose regularly causes a paralysis of the legs, which first appears 10–14 days after poisoning. Until this time the birds appear normal.

2. The levels of the plasma cholinesterase and of the "true" and "pseudo" cholinesterase of the brains and spinal cords of hens killed at varying intervals after poisoning have been compared with those of healthy birds.

3. The pseudo-cholinesterase activity of the plasma and of the brain and spinal cord is markedly diminished as early as one day after poisoning, and in the brain and spinal cord remains at a low level for 10 days at least. The true cholinesterase in these tissues is relatively unaffected.

4. It is concluded that these enzymic changes precede the onset of the demyelination caused by this compound. The significance of these findings is discussed in relation to the general problem of demyelination.

We wish to thank Professor G. Payling Wright for the histological examination of nerve tissue, the Chief Superintendent, Experimental Station, Porton, for a grant for the purchase of birds, Dr. G. R. Webster for the preparation of butyrylcholine perchlorate, and Messrs. Geigy Pharmaceuticals, Ltd., for the gift of the tri-*ortho*-cresyl phosphate. Our thanks are also due to Messrs. D. H. Burchett and D. E. Jarvis for their skilled technical assistance.

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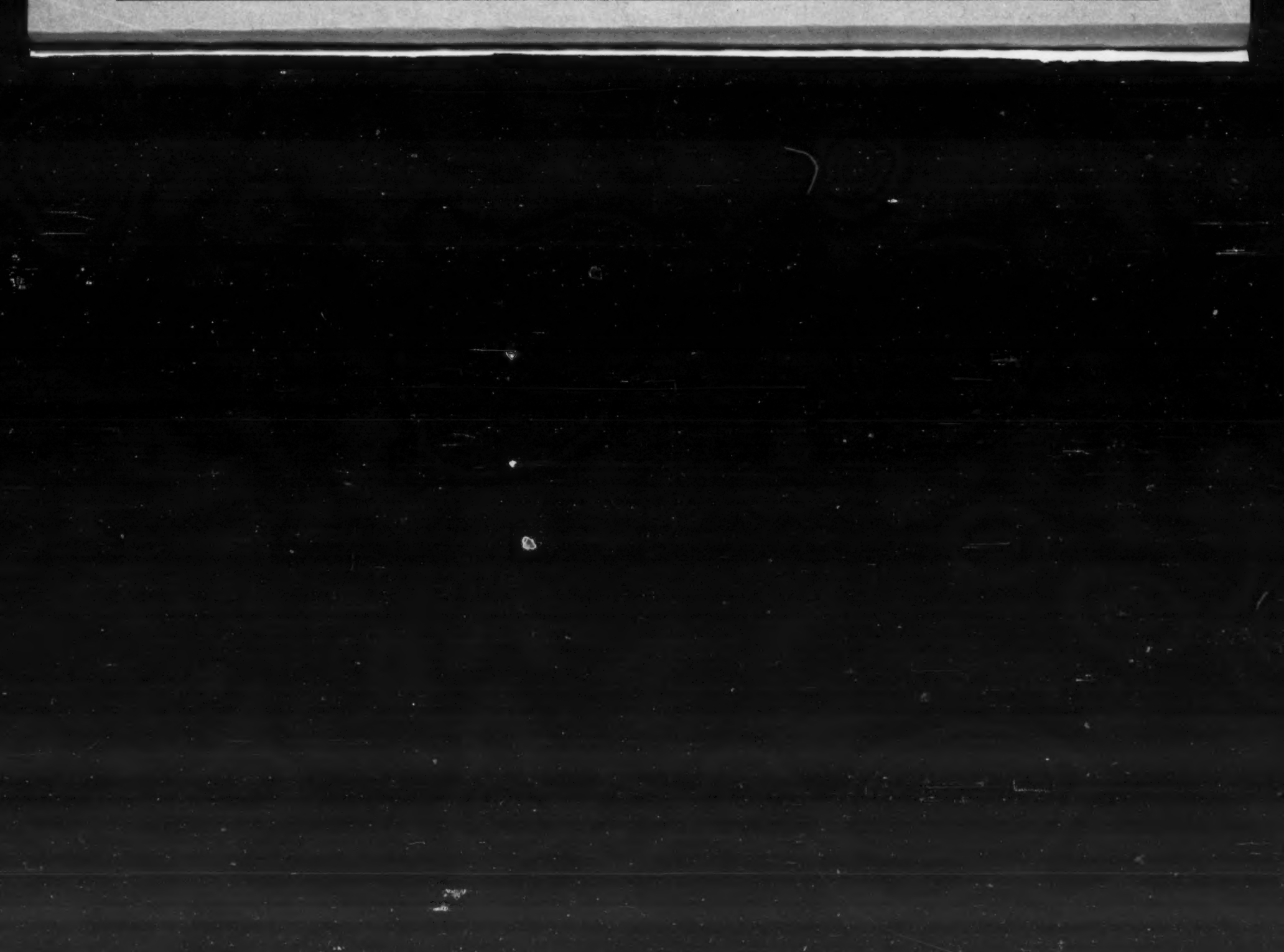

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